Effect of Phosphorylated Organic Compound on the Adsorption of Bovine Serum Albumin by Hydroxyapatite

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The amount of adsorption of bovine serum albumin (BSA) by hydroxyapatite (HAP) increased with a concentration of $CaCl_2$ due to the bridging effect of Ca^{2+} between adsorbate BSA and adsorbent HAP. On the other hand, it decreased remarkably with a concentration of K_2HPO_4. This was explained in terms of the effects of ionic strength and competitive adsorption between inorganic phosphate anion (Pi) and BSA, because BSA is in negatively charged over the examined pHs. A similar effect was observed in the presence of phosphorylated compounds such as phosphoserine, phytate, and phosphorylated polyvinylalcohol. The inhibiting effect of these compounds was stronger than that of their mother compounds (serine, inositol, and polyvinylalcohol). This result shows that phosphate groups bound to the mother compounds interfere with the adsorption of BSA by HAP in the same manner that Pi does. Although the adsorption of BSA was almost irreversible with respect to dilution with water, desorption was performed when these organic phosphorylated compounds were added after the accomplishment of the adsorption of BSA. However, the effective concentration of the phosphorylated compounds for the desorption of BSA was fairly higher than that for the competitive inhibition against the BSA adsorption.

Keywords hydroxyapatite; bovine serum albumin; protein adsorption; phosphoserine; phosphorylated polyvinylalcohol; phosphoprotein

Introduction

Studies on the adsorption of protein by hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) are important from two different viewpoints. The first purpose is to get essential information about the separation/purification of proteins from a mixture by means of HAP column chromatography.[1,2] Proteins on column HAP are usually eluted by a buffer solution of inorganic phosphate salts. Competitive adsorption between inorganic phosphate ion (Pi) and protein occurs at the adsorption sites on the surface of HAP because Pi shows high affinity toward HAP.[3,4] Adsorbed protein is desorbed and eluted, depending on its affinity toward HAP, at a specific Pi concentration/elution volume of the buffer solution.

The second important objective is to understand how mammalian hard tissues (*i.e.*, bones and teeth) are constructed with biological HAP and proteins. Physicochemical studies concerning this field have been those of the interaction of phosphoproteins[5] and enamel proteins[6] with biological HAP. Some researchers have studied the adsorption of human serum albumin onto synthesized HAP as a simplified model system[7-9] because these materials are easily obtainable and thoroghly characterized.

It was shown in the previous paper[10] that transformation of amorphous calcium phosphate (ACP, a precursor of HAP) to crystalline HAP and subsequent crystal growth of HAP were retarded by the adsorption of phosphorylated polyvinylalcohol (Phos. PVA) or phosphoserine (PSer), while the mother compounds, polyvinylalcohol (PVA) and serine (Ser), showed little effects. It was concluded that the retardation was caused through the competition between Pi (one of the constituent ions for HAP) and the phosphate group of the organic compound for the active growth sites on the HAP crystal seed or nucleus. This result is consistent with the view that organic phosphorylated compounds (*i.e.*, mineralization regulator phosphoproteins) regulate the biological mineralization formation of hard tissues.[11,12] However, the fact that many kinds of unphosphorylated compounds are usually distributed over in the neighborhood of premature and mature hard tissues together with phosphoproteins is not sufficiently taken into consideration when the mineralization regulation is discussed through a model system. These compounds should be competing with each other for the binding/interaction sites on the HAP crystal of the hard tissues. The mineralization regulator phosphoprotein is still effective even in the presence of other molecules and ions. This fact means that the adsorption/binding of phosphorylated compounds onto the HAP seed or nuclesus should be preferred over that of the others in a biological body.

In this paper, the adsorption of bovine serum albumin (BSA) on synthetic HAP in the presence of phosphorylated compounds (such as Phos. PVA and PSer) was studied. The significant role of phosphate groups bound to organic compounds for the adsorption/desorption of BSA was stressed, comparing with that of Pi. BSA was taken as a model compound for the non-regulator proteins. Phos. PVA and PSer were regarded as a polymeric and monomeric model compound for the regulator phosphoproteins, because phosphoproteins usually contain ester phosphate groups and/or PSer residues. Comparing the effects of unphosphorylated compounds (PVA and Ser) with those of phosphorylated ones (Phos. PVA and PSer), it becomes possible to examine the differences in the influence between the phosphorylated and unphosphorylated compounds of a given molecular size (*i.e.*, PSer *vs.* Ser, and Phos. PVA *vs.* PVA), and between the low and high molecular compounds at a given ester phosphate concentration (*i.e.*, PSer *vs.* Phos. PVA).

Experimental

Materials HAP used in the present study was the same sample as that used in the previous study.[13] Specific surface area of HAP, S, was determined as $40.78\,m^2/g$ through the N_2 gas adsorption method at 77 K, while it was $47.80\,m^2/g$ according to the CO_2 gas adsorption at 194 K. These measurements were done by means of Belsorp 28 at a laboratory of Bel Japan, Inc. (Osaka). The unit for the adsorption amount of BSA (x_{BSA}) in the present paper is that of the weight of adsorbed BSA per unit mass of HAP (mg/g) instead of per unit surface area of HAP (mg/m^2), because the specific surface area, S, determined by the above two methods

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did not sufficiently coincide with each other.

PVA was Gohsenol NL-05, which was kindly provided by Nippon Gohsei Kagaku Co., Ltd. The viscosity average molecular weight and the degree of polymerization were 1.98×10^4 and 4.50×10^2, as mentioned in the previous paper.[10] Phosphorylation of PVA to Phos. PVA was done according to the method of Kurose *et al.*[14] as mentioned elsewhere.[10]

BSA (fraction V) was purchased from Wako Pure Chemical Industries, Ltd., and another BSA (crystallized and lyophilized) was from Sigma Chemical Company. It was confirmed that the adsorption amounts of BSA on HAP were in fair agreement with each other between the two samples mentioned above.

Other reagents used in the present paper were of analytical grade. These were used without further purification.

Determination of Adsorbed Amount of BSA to HAP All of the adsorption experiments were carried out at 4 °C. Adsorbate BSA solution (20 ml), containing various concentrations of an additive, was prepared immediately before the addition of HAP (300 mg). The HAP suspension was shaken vigorously from time to time. After attaining the equilibrium (at least 4 d at 4°C), the supernatant obtained by filtration or centrifugation was analyzed for BSA. The suspension pH was measured by means of a pH-meter (Yanaco model PH8A) immediately before the filtration or centrifugation. The equilibrium concentration of BSA was determined by colorimetry at 750 nm according to the method of Lowry *et al.*[15]

Determination of Binding Amount of Ca^{2+} to BSA Equilibrium dialysis to determine the binding ratio of Ca^{2+} to BSA was carried out at 4°C for 1 week as follows. Visking cellulose tubing (Union Carbide Co.) containing 10 ml of 0.5 % BSA solution was soaked in a solution (10 ml) of a given concentration of $CaCl_2$. Solution pH was adjusted to 10.0 with NaOH, or to 3.30 with HCl. The pH of an aqueous solution of $CaCl_2$ without any addend was *ca.* 6.4—7.0. After attaining equilibrium, Ca^{2+} concentrations in both compartments were determined by ethylenediamine tetraacetic acid (EDTA) chelatometry with 1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid (*i.e.*, NN indicator) at pH 13. The binding ratio of Ca^{2+} to BSA was determined from the difference in Ca^{2+} concentrations. As the Donnan effect was not taken into consideration, the binding data obtained here were apparent ones.

Treatment of HAP with BSA HAP (3 g) was suspended in an aqueous solution of 0.2 % BSA (200 ml) for 4 d at 4 °C. After centrifugation and subsequent decantation, HAP coated with BSA was washed with 200 ml of distilled water at room temperature. Centrifugation and rinsing were repeated three times. Treated HAP, thus prepared, was freeze-dried and kept at 4°C. The adsorption amount of BSA on HAP before the rinsing was 85.5 mg/g accoring to the determination of BSA in the supernatant through the Lowry method,[15] while the content of BSA after the rinsing was 75.1 mg/g according to the elemental analyses of the treated HAP for carbon and nitrogen (Yanagimoto CHN corder). BSA remaining on the surface of HAP after the treatment was 87.8 %.

Desorption of BSA from the Treated HAP The desorbed amount of BSA was estimated as follows: The treated HAP (0.3 g) was suspended in a solution (20 ml) of a known concentration of phosphorylated or unphosphorylated compound for 4 d at 4 °C. After attaining the desorption equilibrium, BSA in the supernatant was determined by the Lowry method.[15] The percentage of the BSA desorbed from the treated HAP was estimated through the following calculation;

$$(\text{equilibrium concentration of BSA (mg/ml)} \times 20\ (\text{ml})) \div (0.3(\text{g}) \times 75.1(\text{mg/g})) \times 100$$

In the present paper, whether BSA is denatured through adsorption or desorption will not be discussed.

Results

Adsorption of BSA by HAP Adsorption isotherms of BSA to HAP in the presence or absence of NaCl are shown in Fig. 1A. These are of the high affinity type. The binding ratio, x_{BSA}, decreased with an increase in the concentration of NaCl. Figure 1B, rearranged from Fig. 1A, shows the relationship between x_{BSA} and a concentration of NaCl at a given concentration of BSA. The binding ratio leveled off after a steep decrease with an increase in the concentration of NaCl. These results show that the adsorption is mainly electrostatic. It was confirmed that the adsorption was almost irreversible toward dilution with water.

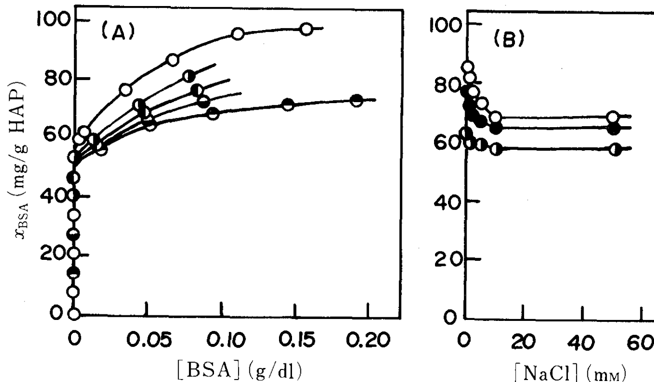

Fig. 1. Binding Ratio of BSA to HAP

A: Relationship between the binding ratio and equilibrium concentration of BSA at a given concentration of NaCl. [NaCl] (mM) = 0 (○), 1 (◑), 2 (◐), 5 (⊖), and 10 (◓). B: Relationship between x_{BSA} and [NaCl] at a given concentration of BSA. [BSA] (g/dl) = 0.10 (◑), 0.15 (●), and 0.20 (○). Equilibrium pH in the supernatant was 7.0—7.2.

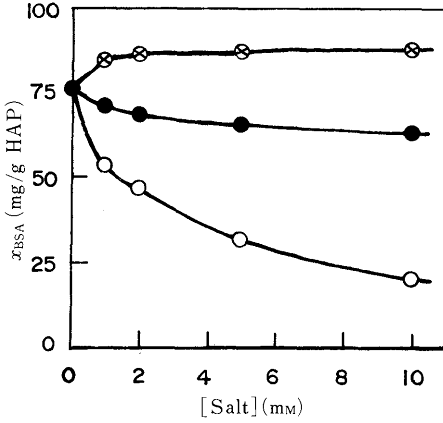

Fig. 2. Effect of $CaCl_2$ and K_2HPO_4 on BSA Adsorption

Concentrations of added BSA and HAP were 0.15 and 1.5 g/dl, respectively. ⊗, $CaCl_2$; ○, K_2HPO_4; ●, NaCl (taken from Fig. 1 B).

Figure 2 shows the relationship between x_{BSA} and the concentration of added $CaCl_2$ or K_2HPO_4 at a given concentration of BSA (= 0.15 g/dl). The adsorption amount of BSA increased with a concentration of added $CaCl_2$ in contrast to the effect of NaCl. On the other hand, it more remarkably decreased with the concentration of added K_2HPO_4 than with that of added NaCl.

The pH of these systems varied with the concentration of the added salt: equilibrium pH in the absence of an additive was 7.05, whereas that at 10 mM of $CaCl_2$, NaCl, and K_2HPO_4 was 6.95, 7.03, and 8.20, respectively, for example. Although the pH was not kept constant, the result mentioned above qualitatively shows that Ca^{2+} accelerates the adsorption of BSA while Pi inhibits it. The effect of pH on the adsorption amount of BSA will be discussed in detail later (see Figs. 6—8).

Binding of Pi and Ca^{2+} to BSA Binding of Pi to BSA was not observed in an aqueous solution of K_2HPO_4 (weakly alkaline). This is probably because both Pi and BSA are negatively charged where the isoionic point for BSA is pH 5.2—5.3.[16]

On the other hand, Ca^{2+} was bound by BSA, as shown in Fig. 3. The binding ratio, $x_{Ca^{2+}}$, increased in the order of pH 6.4, 3.3, and 10.0. It seems reasonable that $x_{Ca^{2+}}$ at pH 10.0 is higher than that at pH 3.3 and 6.4 because the

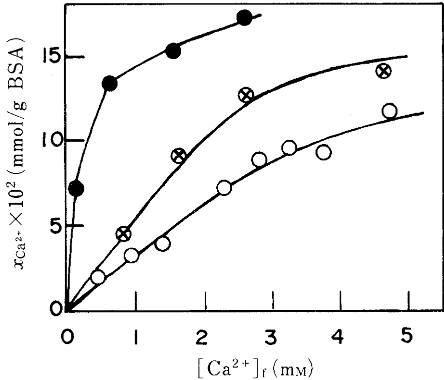

Fig. 3. Binding Isotherm of Ca^{2+} to BSA

●, pH 10.0 ($CaCl_2$ + NaOH); ⊗, pH 3.3 ($CaCl_2$ + HCl); ○, pH 6.4 ($CaCl_2$ only).

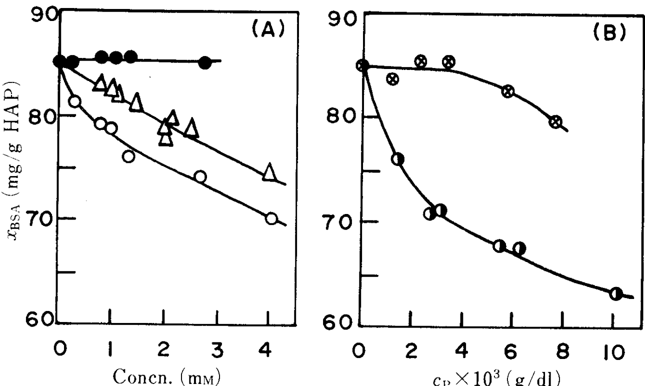

Fig. 4. Effect of Phosphate Groups of the Additives on the Adsorption of BSA

A: Effect of Ser and PSer. ●, Ser (*ca.* pH 7.0); ○, PSer (pH 5.6—7.0); △, NaCl (*ca.* pH 7.1). B: Effect of PVA and Phos. PVA. ⊗, PVA NL-05 (pH 6.9—7.0); ◑, H_2Phos.PVA (pH 6.7—6.9).

Concentrations of added BSA and HAP were kept constant at 0.2 and 1.5 g/dl, respectively. Degree of phosphorylation of Phos. PVA (α) was 8.17%. Abscissa in B shows the polymer concentration converted to that of PVA NL-05 in order to directly compare the effect of Phos.PVA with that of PVA NL-05.

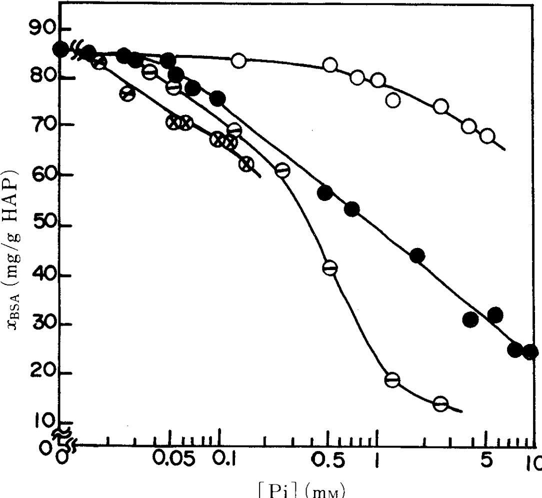

Fig. 5. Relationship between x_{BSA} and Total Concentration of Phosphate Groups of the Additive

○, PSer (pH 5.6—7.0); ●, K_2HPO_4 (pH 7.3—8.2); ⊖, NaPhytate (pH 7.2—9.0); ⊗, H_2Phos.PVA (α = 8.17%, pH 6.7—6.9).

Concentrations of added BSA and HAP were as in Fig. 4.

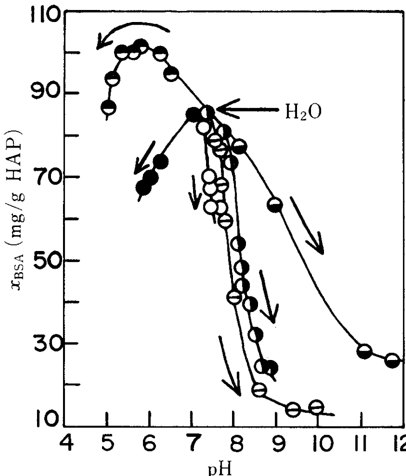

Fig. 6. Relationship between x_{BSA} and Equilibrium pH

The solution pH was adjusted by HCl (◓), NaOH (◒), PSer (acid form, ●), Na_2Phos.PVA (α = 8.17%, ○), NaPhytate (⊖), and K_2HPO_4 (◑). Concentrations of added BSA and HAP were as in Fig. 4. The sign of H_2O with the arrow mark shows the point where no additives were added to the systems of HAP + BSA + water (x_{BSA} = 85.0 mg/g).

negative charge of BSA is higher at pH 10 than at pH 3.8 and 6.4. But it seems strange that $x_{Ca^{2+}}$ at pH 3.3 is higher than that at pH 6.4. However, the latter result may be explained as follows: owing to the conformation change of BSA (*i.e.*, N–F transition and acid expansion) at acidic pH,[16] the BSA molecule expands and exposes more binding sites to cations (such as Ca^{2+} and H^+) at acidic pH than at neutral pH.

It has already been shown elsewhere[17] that Ca^{2+} is easily bound to the surface of HAP. Calcium ion, therefore, bridges BSA and HAP by virtue of its high affinity for both of them. This is the reason why x_{BSA} increases in the presence of $CaCl_2$ (see Fig. 2). On the other hand, Pi shows high affinity for HAP[3] but not for BSA, as mentioned above. Therefore, electrostatic repulsion between negatively charged BSA and Pi adsorbed on HAP increases with an increase in the concentration of K_2HPO_4, resulting in more of a decrease in x_{BSA} than in the presence of NaCl (see ○ and ● in Fig. 2). As the affinity of univalent cations, K^+ and Na^+, for BSA and HAP is weak,[3] the bridging effect by them is negligible.

Effect of Phosphate Group on the BSA Adsorption The adsorbed amount of BSA, x_{BSA}, was not affected by added Ser in the concentration range of 0—3 mM, while it decreased with a concentration of added PSer, as shown in Fig. 4A. The effect of PSer was more conspicuous than that of NaCl. The value of x_{BSA} slightly decreased with a concentration of PVA NL-05, while it decreased noticeably in the presence of H_2Phos. PVA (acid form of Phos. PVA), as shown in Fig. 4B. These results show that phosphate groups bound to the mother compounds (Ser and PVA) strongly inhibit the adsorption of BSA by competition for the adsorption sites on HAP,[10] in the same manner as that of Pi (see Fig. 2). Strictly speaking, the effect of pH on the binding ratio should be taken into consideration, as is mentioned later (see Figs. 6 and 7).

Figure 5 shows the binding ratio, x_{BSA}, as a function of the concentration of the phosphate group. The binding ratio decreased with the concentration in the order of PSer, K_2HPO_4, sodium phytate (NaPhytate), and H_2Phos. PVA. The effect of H_2Phos. PVA was remarkable even at low

concentrations whereas the other low molecular compounds could scarcely exhibit any effect. This is owing mainly to fact that Phos. PVA is a polymeric compound.[10)]

Effect of pH on x_{BSA} Figure 6 shows x_{BSA} as a function of solution pH which was prepared by the concentration of an additive itself. The arrow mark indicates the direction to which the additive concentration increases. The value of x_{BSA} attained its maximum at around pH 6 (see ◓). This is almost the mid-point of the isoionic point of BSA (pH 5.2—5.3)[16)] and the isoelectric point of HAP (*ca.* pH 7.0),[7,18)] although the point of zero charge of HAP is reported as pH 8.5 through a potentiometric titration method.[19)]

The pH decreased with a concentration of PSer because it was of the acid form in the present paper. The x_{BSA} in the presence of PSer monotonously decreased with a decrease in solution pH (● in Fig. 6) and/or with an increase in the concentration (○ in Fig. 5) in contrast with the effect of HCl at the same pH range (see ◓ in Fig. 6). Adsorbed hydrogen ion offers a positive charge to HAP and accelerates the adsorption of BSA which still remains negatively charged (solution pH > isoionic point). In the case of acidic PSer, however, both the hydrogen ion and PSer are adsorbed by HAP. PSer adsorbed on HAP through the phosphate group inhibits the adsorption of negatively charged BSA by electrostatic repulsion.

The solution pH increased with a concentration of Phos. PVA, because that used here was of the disodium salt (Na_2Phos. PVA) which was prepared by neutralization of H_2Phos. PVA with NaOH. The x_{BSA} decreased with an increase in pH which was prepared with various concentrations of K_2HPO_4, NaPhytate, or Na_2Phos. PVA. The effect of the additive on x_{BSA} at a given pH was in the order of NaOH, K_2HPO_4, NaPhytate, and Na_2Phos. PVA. This sequence is in agreement with that obtained from Fig. 5. Phos. PVA showed the most pronounced effect irrespective of Na_2- or H_2-form.

Effect of Ester Phosphate at a Given pH As mentioned above, x_{BSA} was affected by pH, ionic species of the additives, and their concentrations. The interrelation among these was somewhat complicated. It is, therefore, necessary for the pH to be kept constant in order to obtain clearer information about the effect of the concentration of an additive on x_{BSA}.

Figure 7 shows the relationship between x_{BSA} and the concentration of the phosphate group of esters at pH 7.2 adjusted by 0.1 M sodium barbital and HCl. Sodium barbital (sodium 5,5-diethylbarbiturate) was used as a buffering agent because it is almost indifferent toward HAP. It was not considered in the present paper whether the inherent physicochemical properties of the BSA molecule might or might not be affected by the addition of sodium barbital. The ionic strength, needless to say, increased with the addition. In any event, the x_{BSA} decreased from 85 mg/g (see the ordinate in Fig. 5) to 78 mg/g (see the ordinate in Fig. 7) by the addition of 0.1 M sodium barbital.

The sequence of the additives at pH 7.2 with respect to the effect on a decrease in x_{BSA} was PSer, phytate, and Phos. PVA. This is the same order as that obtained in Figs. 5 and 6. Therefore, it can be concluded that Phos. PVA is the most effective for inhibiting the adsorption of BSA to HAP irrespective of the pH.

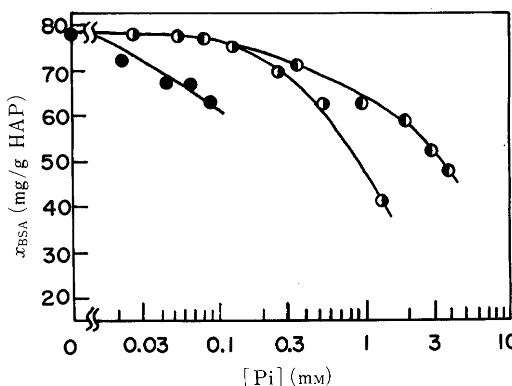

Fig. 7. Relationship between x_{BSA} and Concentration of Phosphate Group of the Additive at pH 7.2

●, Phos.PVA (α = 8.17%); ◑, NaPhytate; ◐, PSer. The solution pH (7.2) was buffered by 0.1 M sodium barbital and HCl.

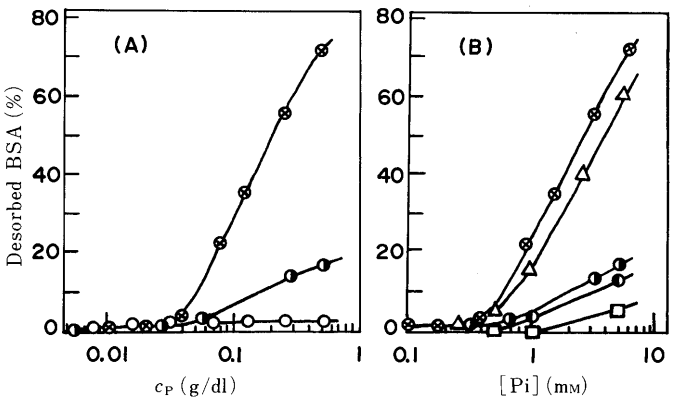

Fig. 8. Desorption of BSA from the Treated HAP

A: Relationship between percentage of the desorbed BSA and concentration of the added polymer. Concentration of Phos.PVA on the abscissa in (A) was shown after conversion to that of PVA NL-05 in order to compare the effect of Phos.PVA with that of PVA NL-05.

B: Relationship between percentage of the desorbed BSA and concentration of phosphate groups of the additive. ⊗, Na_2Phos.PVA (pH 8—9); ◑, H_2Phos.PVA (pH 5—7); ○, PVA NL-05 (*ca.* pH 7); △, NaPhytate (pH 7—8); ◐, K_2HPO_4 (pH 6—8); □, PSer (pH 5—7). The degree of phosphorylation of Phos.PVA (α) used for this measurement was 5.2%.

Desorption of BSA from the Treated HAP The percentage of the amount of BSA desorbed from the treated HAP was shown in Fig. 8 as a function of the additive concentration. PVA NL-05 was almost ineffective for the BSA desorption, while phosphorylated PVA NL-05 (*i.e.*, Phos. PVA) was remarkably effective. The effect on the desorption of BSA was stronger in Na_2Phos. PVA (pH 8—9) than in H_2Phos. PVA (pH 5—7) even through the degree of phosphorylation, α, was the same. This effect is explained in terms of the increase in electrostatic repulsion with an increase in pH between BSA and HAP (see Fig. 6) and between BSA and the esterified phosphate groups of the adsorbed Phos. PVA. The ionized phosphate group could easily be adsorbed on HAP, while the adsorbed BSA is simultaneously desorbed from HAP instead.

According to Fig. 8B, the sequence of the additives with respect to the effect on the desorption was Na_2Phos. PVA, Naphytate, H_2Phos. PVA, K_2HPO_4, and PSer. This order is quite similar to that with respect to the inhibition of the BSA adsorption (see Figs. 4, 5, and 7). However, the available concentration for the desorption (Fig. 8) was fairly

higher than that for the adsorption inhibition (Figs. 4, 5, and 7). This result means that the desorption of BSA after the adsorption is more difficult than the competitive inhibition against the adsorption of BSA to HAP. The effect of NaCl on the desorption was very weak. Only 2% of the adsorbed BSA was desorbed by the addition of *ca.* 100 mM NaCl (not shown). This result of desorption is in contrast with that of adsorption inhibition by NaCl (Figs. 1B and 2). These facts again show the significance of the role of the phosphate groups but not of the ionic strength.

Discussion

The geometrical shape of native BSA is ellipsoidal, the size of which is reported as 4.16 nm × 14.09 nm.[16,20] Therefore, the projected area with the BSA long axis parallel to the surface (*i.e.*, side-on mode) is 4.60×103 Å^2/molecule, while that with the long axis perpendicular to the surface (*i.e.*, end-on mode) is 1.36×10^3 Å^2/molecule. According to the adsorption isotherm in Fig. 1, the maximum amount of the adsorbed BSA was *ca.* 95.0 mg/g. Therefore, the area occupied by a molecule of BSA at maximum adsorption is 4.73×10^3 Å^2/molecule for $S = 40.78$ m^2/g (determined by N_2 gas adsorption), whereas 5.54×10^3 Å^2/molecule for $S = 47.80$ m^2/g (determined by CO_2 gas adsorption), where the relative molar mass of BSA was regarded as 6.63×10^4 [16] for the calculation. The occupied area calculated in this way is slightly larger than the projected one with the side-on mode of BSA. Therefore, it can be concluded that the BSA molecule is attached to the HAP surface in a manner of the side-on mode of monolayer in the concentration range studied in the present paper.

According to the crystallographic data for HAP, it is hexagonal, and geometrical parameters for the unit cell are $a = b = 9.42$ Å, $c = 6.88$ Å, and $\beta = 120$ degrees.[21,22] The area of the unit crystal face of (001), (100), and (010) is calculated as 77.0, 64.9, and 64.9 Å^2, respectively. Therefore, each BSA molecule at maximum adsorption covers 61—85 faces of the unit lattices of the HAP crystal. Strictly speaking, however, that depends on which crystal faces of HAP are dominantly exposed to the solution.

BSA is adsorbed mainly through electrostatic attraction between the carboxylate group of BSA and calcium ion exposed on the surface of HAP and/or through ion-exchange between the carboxylate group of BSA and the surface phosphate or hydroxyl group on HAP.[7,23] Adsorbed BSA comes in contact with a lot of the lattice faces and constituent ions on the surface of HAP, as mentioned above, resulting in the multi-site and high-affinity type adsorption.

When an ionic additive was added to the solution, the adsorption amount of BSA decreased due to the electrostatic shielding effect (*i.e.*, the effect of ionic strength) and to the competitive adsorption between them. The phosphate group of the additive mainly contributes to the competition/inhibition against the BSA adsorption, because the phosphate group competes with carboxylate groups of BSA for the positively charged adsorption sites and/or the anion-exchanging sites on the surface of HAP.[3,4,10,24] A higher concentration of the additive was required for the desorption (Fig. 8) than for the competitive inhibition (Figs. 4—7). This is because BSA has tightly contacted with HAP by virtue of the multi-site adsorption prior to the addition of the desorbing agent.

The effects of phytate and Phos. PVA were more remarkable than those of PSer and Pi over the concentration range studied. The former compounds are polyfunctional with respect to phosphate groups. Some of the phosphate groups may be bound to the adsorption sites on HAP, while the other may remain unattached but localize close to the surface of HAP. They repel the negatively charged BSA which is approaching to and/or covering the surface of HAP. In addition to the electrostatic effect, protruding polymer loops and/or tails of adsorbed Phos. PVA prevent the adsorption of BSA by virtue of the steric hindrance. Therefore, Phos. PVA is the most conspicuous in inhibiting the adsorption of BSA, when compared to a given concentration of phosphate groups. It is also effective in peeling off the adsorbed BSA from the surface of HAP, resulting in a strong desorbing agent for BSA.

In order to play the role of regulator in biological calcification, the phosphoproteins, in preference to other inorganic/organic compounds present in body fluids, should be in contact with the crystal seed and/or nuclei of the biological HAP. According to the present study, Phos. PVA and PSer (simplified model compounds for a regulator phosphoprotein)[10] effectively repelled BSA (a model compound for ordinary proteins) from the surface of HAP. It was thus shown by using the model compounds how the regulator phosphoproteins effectively perform the role even in the presence of other proteins and organic compounds which are surrounding the biological HAP seed and/or nuclei.

In application to separation chemistry, phytate might be more useful than Phos. PVA as a desorbing agent for the proteins which are stickily adsorbed on HAP in the column. A protein desorbed from HAP but mixed with phytate might be easily purified by means of dialysis, while the separation of the protein from Phos. PVA seems rather difficult because the latter is a polymer impermeable to a dialyzing membrane.

Acknowledgement The research described herein was supported in part by the 1989 Takeda Science Foundation (Osaka, Japan).

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Synthetic Studies of Indoles and Related Compounds. XXVII.[1] A New Synthesis of Crenatine from Ethyl Indole-2-carboxylate[2]

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Crenatine (1a), which is a member of a new class of β-carboline alkaloids having an oxygen functionality at the 4-position, was synthesized starting from ethyl 1-benzylindole-2-carboxylate (12a) *via* cyclization of an elaborated C_2-substituent to the 3-position of the indole nucleus and aluminum chloride-catalyzed debenzylation of the protected indolic nitrogen. 1-Ethyl-4-hydroxy-9-methyl-β-carboline (26b), a positional isomer of crenatine with regard to the methyl group, was also synthesized through the same methodology.

Keywords crenatine; ethyl indole-2-carboxylate; indole; β-carboline; cyclization; debenzylation; methylation

Recently a number of 4-oxygenated β-carboline alkaloids (1) have been isolated from Simarubaceae by Ohmoto *et al.*[3a-d] and others.[3e-h] Representative compounds are shown in Table I. Although usual 4-unsubstituted β-carbolines have long been known, the 4-oxygenated ones constitute a new class of β-carbolines. Among them, 4-hydroxy-β-carboline-1-carbaldehyde (**1b**) has been reported[4] to have antitumor and xanthine oxidase-inhibitory activities. However, the biological activities of other compounds have not been examined, probably because of the limited amounts that could be isolated from natural sources. There have been only a few synthetic examples reported by Cook and co-workers, who synthesized crenatine[5] (**1a**) and 1-methoxycanthin-6-one[6] (**1g**) *via* 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation at the 4-position of the β-carboline nucleus as a key step. We now report[2] details of a new synthesis of crenatine (**1a**), which should provide a general synthetic route to these 4-oxygenated β-carboline alkaloids (**1**).

TABLE I. Naturally Occurring 4-Oxygenated-β-carbolines

1a—f

	R_1	R_2
1a	Me	Et (crenatine)
1b	H	CHO
1c	Me	CO_2Me
1d	Me	COMe
1e	Me	$CH(OH)CH_2OH$
1f	Me	$CH{=}CH_2$

1g: **1h**:

Trial of Cyclization from the 3- to the 2-Position of the Indole Nucleus for β-Carboline Synthesis At first, we planned to synthesize 4-oxygenated β-carbolines (**1**) *via* the Bischler–Napieralski reaction.[7] However, a direct application of the Bischler–Napieralski reaction to the 3-acylamide (**2**) has been reported[8a,b] to give an oxazole (**4**), but not the desired cyclization product (**3**). As this result shows that the amide part reacted with the 3-acyl part but not with the C_2-position, we tried protection of the 3-acyl group. The conversion of the 3-acylamide (**2**) into the ketal (**5**) was unsuccessful, but the 3-acylamide (**2**) was converted into the thioketal amide (**6**) in reasonable yield. The reaction of the thioketal amide (**6**) with phosphorus oxychloride ($POCl_3$) did not give the expected cyclization product (**7**), but abnormally gave 3-propionylindole (**8**) as a sole product. This compound (**8**) is presumably formed as follows. The amide (**6**) is transformed to the imino phosphate (**9**), which cyclizes toward the C_3-position to yield the spiro intermediate (**10**). In this intermediate (**10**), the older bond of the two at the C_3-position is cleaved to provide a cation (**11**), which is hydrolyzed to 3-propionylindole (**8**). Bischler–Napieralski reaction of indoles was suggested[9] to proceed through a spiro intermediate such as **10** rather than by direct attack at the C_2-position. In the present case, as cleavage of the older bond at the C_3-position in the intermediate (**10**), leading to a more stable cation (**11**), is favored over rearrangement of the newer bond at the C_3-position, a β-carboline skeleton would not be formed. This mechanism has been supported by Cook *et al.*[6a]

Cyclization of the 2-Substituent to the 3-Position to Obtain the β-Carboline Skeleton Thus, we developed a new methodology for construction of 4-oxygenated β-carboline using ethyl indole-2-carboxylates (**12**), on the basis of our studies on the synthetic chemistry of ethyl indole-2-carboxylates.[1,10] The present strategy involves the use of the 2-carboethoxy group of ethyl indole-2-carboxylates (**12**) as a one-carbon unit, and cyclization of the elongated C_2-substituent to the nucleophilic 3-position. It is an advantage that the cyclization also results in the introduction of an oxygen functionality at the 4-position of the β-carboline skeleton. However, this cyclization reaction has a problem as to the direction of cyclization. That is, Johnson *et al.*[11] reported that cyclization of the

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Chart 1

synthetic route

Johnson's results

20 : 80

Chart 2

2-acyl compound (**15**) gave the C_3-cyclized product (**16**) as a minor product, and the C_1-cyclized product (**17**) as the major one. Thus, we used ethyl 1-benzyl-1*H*-indole-2-carboxylate (**12a**) as a substrate in order to protect the 1-position. The reason for use of the benzyl group is that the electron-donating 1-benzyl group should make the C_3-position more nucleophilic and that the benzyl group can be removed at any time after cyclization by means of a new and mild method which we had developed for debenzylation of 2-acylindoles.[12]

The 1-benzylindole (**12a**) was allowed to react with ethyl propionate under Claisen condensation conditions to give the keto-ester (**18a**). The keto-ester (**18a**) was, without purification, treated with sulfuric acid to give 1-benzyl-2-propionyl-1*H*-indole (**19a**) (ketone degradation). Conversion of **19a** into the glycinate (**22a**) by treatment with ethyl glycinate *via* formation of the Schiff's base or reductive amination was unsuccessful. The introduction of a nitrogen functionality into the 2-propionylindole (**19a**) to obtain the formamide (**21a**) was achieved by employing the Leuckart reaction under high temperature and pressure, but the yield was variable (8—54%), and the autoclaving procedure was inconvenient. For improvement of this step, the propionylindole (**19a**) was converted to the oxime (**20a**) in a usual manner. The oxime (**20a**) was separable into two geometrical isomers [(*E*)- and (*Z*)-**20a**] but their configuration was not

Chart 3

determined. The oxime [**20a**, a mixture of (*E*)- and (*Z*)-] was reduced with sodium borohydride–titanium tetrachloride and the resulting amine was formylated with ethyl formate to give the same formamide (**21a**). This reaction was better than the above-mentioned Leuckart reaction from the viewpoints of easy handling and average yield. The *N*-alkylation of the formamide (**21a**) with ethyl chloroacetate under basic conditions smoothly gave the glycinate (**22a**). Although the glycinate (**22a**) showed a clear single spot on thin layer chromatography (TLC) and a sharp melting point, ^{1}H-nuclear magnetic resonance (^{1}H-NMR) of **22a** showed apparently an equimolar mixture of two isomers (see the experimental section). This can be explained in terms of rotational isomerism due to the formamide moiety. The glycinate (**22a**) was then cyclized with polyphosphoric acid (PPA) to give the cyclic ketone (**24a**).

The cyclization toward the 3-position is inevitable, as the 1-position is blocked by the benzyl group in the glycinate (**22a**). In the case of the cyclization of the 1-unsubstituted substrate (**15**), a mixture of two cyclized products (**16** and **17**) has been obtained.[11] Thus, we were interested in the cyclization direction for the corresponding NH-compound (**22c**) in comparison with that of **15**. Debenzylation of the *N*-benzyl glycinate (**22a**) to prepare the NH-glycinate (**22c**) was unsuccessful. Thus, 1-benzyl-2-propionyl-1*H*-indole (**19a**) was debenzylated to the corresponding NH-compound (**19c**). The reductive amination of **19c** with ethyl glycinate was also unsuccessful. Then 2-propionylindole (**19c**) was treated in the same way as the corresponding 1-benzyl derivative (**19a**) to give the NH-formamide (**21c**). The next alkylation of **21c** with ethyl chloroacetate, however, gave only the 1-alkylated compound (**23**) with recovered starting material (**21c**) (40%), but not the desired glycinate (**22c**). These unsuccessful results show that the protection of indolic NH is appropriate for the present route. The problem of cyclization in the NH-indole series remains to be solved.

The hydrolysis of the cyclic ketone (**24a**) with hydrochloric acid gave the NH-compound (**25a**), which was in turn aromatized with 10% palladium on carbon to give the 4-hydroxy-*β*-carboline (**26a**) in a reasonable yield. The methylation of the 4-hydroxy-*β*-carboline (**26a**) was carried out with diazomethane or dimethyl sulfate–base to give the desired methyl ether (**27**). But the best yield was only 39%, although various conditions were examined. The final debenzylation process to obtain crenatine (**1a**) was achieved[13] by application of our method[12] using aluminum chloride in anisole. This synthetic crenatine was identical with the natural one[3b,g] as judged from the ^{1}H-NMR and infrared (IR) spectra, and mixed melting point determination.

Although the ^{1}H-NMR spectrum of our synthetic sample is identical with that of the natural product, Cook's spectrum[5] seems to be slightly different, as shown in Table II. Although the melting point of their sample was the same as that of the natural product, we considered that their sample might be 1-ethyl-4-hydroxy-9-methyl-*β*-carboline (isocrenatine) (**26b**), which might be formed by unexpected *N*-methylation of 4-demethyl crenatine (**28**) in place of the

TABLE II. Comparison of ¹H-NMR Data for Three Kinds of Crenatine (in $CDCl_3$)

¹H-NMR ($CDCl_3$) δ (ppm)	Natural **1a** (Sánchez and Comin)[3g]	Synthetic **1a** (Ours)	Synthetic **1a** (Cook *et al.*)[5]	Isocrenatine (**26b**)
OMe	4.10 (3H, s)	4.08 (3H, s)	4.42 (3H, s)	—
N-Me	—	—	—	4.12 (3H, s)
$CH_2C\underline{H}_3$	1.40 (3H, t, J=7 Hz)	1.42 (3H, t, J=7.0 Hz)	1.50 (3H, t, J=8 Hz)	1.47 (3H, t, J=7.6 Hz)
$C\underline{H}_2CH_3$	3.13 (2H, q, J=7 Hz)	3.06 (2H, q, J=7.0 Hz)	3.35 (2H, q, J=8 Hz)	3.42 (2H, q, J=7.6 Hz)
C_3-H	8.00 (1H, s)	7.93 (1H, s)	8.55 (1H, s)	8.18 (1H, s)
C_5-H	8.37 (1H, d, J=7.5 Hz)	8.27 (1H, d, J=8.0 Hz)	8.95 (1H, d, J=8.0 Hz)	8.52 (1H, d, J=8.2 Hz)
mp (°C)	177—179	180—182	174	276—282 (dec.)

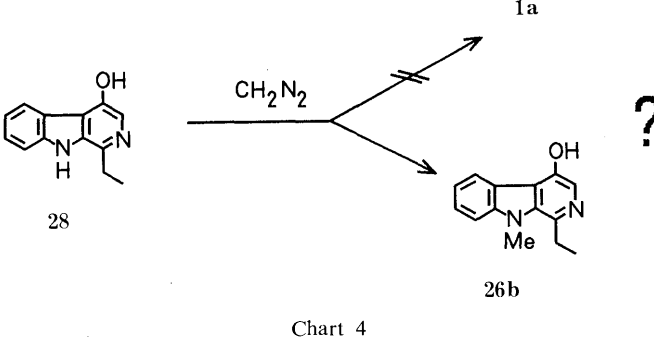

Chart 4

expected *O*-methylation at their final methylation step with diazomethane[5] (Chart 4). This would be consistent with the facts that our 4-hydroxy compound (**26a**) had unexpectedly low reactivity for *O*-methylation and that an *N*- and *O*-bifunctionalized compound[14] was reported to react with diazomethane preferentially at the *N*-position.

In order to clarify this question, we planned a synthesis of isocrenatine (**26b**). The synthesis was carried out in the same way as that of crenatine (**1a**), starting from ethyl 1-methyl-1*H*-indole-2-carboxylate (**12b**), as shown in Chart 3 (b-series). The sequence of reactions from **12b** to **26b** could be carried out in a similar manner to that of the a-series (from **12a** to **26a**). A special feature of the b-series is that the ketone degradation of **18b** to 1-methyl-2-propionyl-1*H*-indole (**19b**) by refluxing of **18b** in a mixture of H_2SO_4 and acetic acid gave a mixture of the desired **19b** and 1-methyl-3-propionyl-1*H*-indole, formed by rearrangement of the 2-propionylindole (**19b**). Milder conditions gave the desired 2-acyl one (**19b**) as the major product. The melting point [mp 276—282 °C (dec.)] and ¹H-NMR data of thus prepared isocrenatine (**26b**), shown in Table II, and its solubility in organic solvents are very different from those of crenatine (**1a**). This result clearly shows that Cook's sample was crenatine (**1a**), not isocrenatine (**26b**).

Conclusion

In this paper we present a synthesis of crenatine by a new methodology which involves the use of ethyl 1-benzyl-1*H*-indole-2-carboxylate (**12a**) as the starting material and cyclization from the 2- to the 3-position of the indole nucleus. This type of cyclization has not been much employed for β-carboline synthesis[11] and thus represents a useful supplement to known methods (cyclization from the 3- to the 2-position). During the above sequence, we found that the aluminum chloride-catalyzed debenzylation[12] method for 2-acylindoles developed by us was also effective for β-carboline. We are now investigating the application of this methodology to general synthesis of 1-substituted 4-oxygenated β-carbolines (**1**) and will report the results in the near future.

Experimental

All melting points were measured on a micro melting point hot stage apparatus (Yanagimoto) and are uncorrected. IR spectra were recorded on a Shimadzu IR-400 spectrometer (in Nujol, unless otherwise stated). ¹H-NMR spectra were measured on Hitachi R-24B (60 MHz), Hitachi-R 900 (90 MHz), JEOL-4H-100 (100 MHz), and JEOL GX-400 (400 MHz) spectrometers in deuteriochloroform unless otherwise stated, with tetramethylsilane as an internal reference. The data at 60 MHz were recorded, unless otherwise stated. The assignments of NH signals of indoles were confirmed by disappearance of the signals after addition of deuterium oxide, and the protons of the 3-position were identified at the same time, by observing that the broad singlet or doublet signal changed to a sharp singlet signal. Mass spectra (MS) were measured on JEOL JMS-01-SG-2 and JEOL JMS-D 300 spectrometers with a direct inlet system. Column chromatography was carried out over silica gel. For column chromatography, Silica gel 60 (70—230 mesh ASTM, Merck, unless otherwise stated), and for TLC, Silica gel 60 F_{254} (Merck) were used. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; br, broad; dif, diffused; Ar: aromatic; BP, base peak.

***N*-[2,2-Ethylenedithio-2-(1*H*-indol-3-yl)ethyl]propionamide (6)** Ethanedithiol (1.7 ml, 20.3 mmol) and boron trifluoride etherate (2.5 ml, 20.3 mmol) were added to a solution of *N*-[2-oxo-2-(1*H*-indol-3-yl)-ethyl]propionamide[8a,b] (**2**) (0.800 g, 3.47 mmol) in acetic acid (15 ml). The mixture was stirred at room temperature for 44 h, then poured into water and extracted with ethyl acetate. The organic layer was washed with 5% NaOH, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was purified by column chromatography using benzene–ethyl acetate (10 : 1) as a solvent to give the title compound (**6**) (640 mg, 60%). Recrystallization from ethyl acetate–hexane gave colorless needles, mp 153—154 °C. *Anal.* Calcd for $C_{15}H_{18}N_2OS_2$: C, 58.79; H, 5.92; N, 9.14. Found: C, 58.60; H, 5.95; N, 8.96. IR ν_{max} cm^{-1}: 3270 (NH), 1650 (C=O). ¹H-NMR (DMSO-d_6) δ: 0.97 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 2.13 (2H, q, J=7.5 Hz, $OC\underline{H}_2CH_3$), 3.45 (4H, s, $SC\underline{H}_2C\underline{H}_2S$), 3.97 (2H, d, J=6.0 Hz, $CC\underline{H}_2N$), 6.9—8.0 (6H, m, Ar-H and NH), 10.9 (1H, brs, NH). MS *m/z*: 306 (M^+, 3%), 220 (BP).

1-(1*H*-Indol-3-yl)-1-propanone (8) $POCl_3$ (0.077 ml, 0.8 mmol) was added to a solution of the thioketal (**6**) (50 mg, 0.16 mmol) in acetonitrile (0.5 ml), and the mixture was stirred at room temperature for 3 h and then at 50 °C for 1.3 h under an argon atmosphere. The reaction mixture was then poured into water and extracted with ether. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue (36 mg) was purified by column chromatography using benzene–ethyl acetate (10 : 1) to give the title compound (**8**) (23 mg, 81%). Recrystallization from benzene gave colorless prisms, mp 173—174 °C. This compound was identical with an authentic sample, (lit.[15] mp 171—173 °C). IR ν_{max} cm^{-1}: 3150 (NH), 1630 (C=O). ¹H-NMR δ: 1.26 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 2.92 (2H, q, J=7.5 Hz, $C\underline{H}_2CH_3$), 7.2—7.6 (3H, m, $C_{5,6,7}$-H), 7.86 (1H, difd, J=3.0 Hz, C_2-H), 8.3—8.5 (1H, m, C_4-H), 9.0 (1H, brs, NH). MS *m/z*: 173 (M^+, 31%), 144 (BP).

1-(1-Benzyl-1*H*-indol-2-yl)-1-propanone (19a) A 1.6 M solution of *n*-butyllithium (67 ml, 0.11 mol) in hexane and a solution of hexamethylphosphoramide (HMPA) (18.9 ml, 0.11 mol) in dry tetrahydrofuran

(THF) (10 ml) were added to a solution of diisopropylamine (15.2 ml, 0.11 mol) in dry THF (15 ml) at −78 °C under an argon atmosphere. Ethyl propionate (12.7 ml, 0.11 mol) was added portionwise to the above solution, and the resulting solution was stirred at the same temperature for 10 min. To this solution, a solution of ethyl 1-benzyl-1*H*-indole-2-carboxylate (**12a**) (10.0 g, 35.8 mmol) in dry THF (20 ml) was added portionwise. The mixture was allowed to reach room temperature and stirred for 20 min, then poured into H_2O, neutralized with aqueous NH_4Cl, and extracted with ether. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue (37.2 g) was column-chromatographed using hexane–ethyl acetate (40 : 1) to give the starting material (**12a**) (3.65 g) and ethyl 2-methyl-3-oxo-3-(1-benzyl-1*H*-indol-2-yl)propionate (**18a**) (12.5 g, contaminated with HMPA) as an oil. ¹H-NMR δ: 1.12 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.45 (3H, d, J=7.0 Hz, $CHC\underline{H}_3$), 4.10 (2H, q, J=7.0 Hz, $OC\underline{H}_2CH_3$), 4.35 (1H, q, J=7.0 Hz, $COC\underline{H}CH_3$), 5.84 (2H, s, $NC\underline{H}_2Ph$), 6.9—7.5 (9H, m, Ar-H), 7.6—7.9 (1H, m, C_4-H). MS m/z: 335 (M^+, 60%), 91 (BP).

This β-keto ester (**18a**) (12.5 g) was, without further purification, added to a solution of concentrated H_2SO_4 (8.8 ml) in a mixture of water (44 ml) and acetic acid (66 ml), and the mixture was refluxed for 1.6 h. The reaction mixture was poured into water and extracted with methylene chloride. The organic layer was washed with 5% aqueous $NaHCO_3$, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The crystalline residue (7.11 g, 76%) was recrystallized from benzene–hexane to give colorless prisms (6.84 g, 73%), mp 64—66 °C. Further recrystallization from the same solvent gave an analytical sample as colorless prisms, mp 65.5—66 °C. *Anal.* Calcd for $C_{18}H_{17}NO$: C, 82.10; H, 6.51; N, 5.32. Found: C, 81.79; H, 6.49; N, 5.33. IR ν_{max} cm⁻¹: 1670 (C=O). ¹H-NMR δ: 1.16 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.96 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 5.81 (2H, s, $NC\underline{H}_2Ph$), 6.9—7.5 (9H, m, Ar-H), 7.6—7.8 (1H, m, C_4-H). MS m/z: 263 (M^+, 40%), 91 (BP).

1-(1-Methyl-1*H*-indol-2-yl)-1-propanone (19b) Ethyl 1-methyl-1*H*-indole-2-carboxylate (**12b**) (4.07 g) was allowed to react under the same reaction conditions as used for the benzyl compound (**12a**). Ethyl 2-methyl-3-oxo-3-(1-methyl-1*H*-indol-2-yl)propionate (**18b**) (4.58 g, 88%) was obtained as a yellow oil. IR ν_{max} cm⁻¹: 1735, 1663 (C=O). ¹H-NMR δ: 1.18 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.49 (3H, d, J=7.0 Hz, $CHC\underline{H}_3$), 4.01 (3H, s, NCH_3), 4.12 (2H, q, J=7.0 Hz, $OC\underline{H}_2CH_3$), 4.19 (1H, q, J=7.0 Hz, $C\underline{H}CH_3$), 6.9—7.8 (5H, m, Ar-H). The β-keto ester (**18b**) was used for the following reaction without further purification.

The β-keto ester (**18b**) was stirred in a mixture of 30% aqueous H_2SO_4 and acetic acid (2 : 1 v/v) at 80 °C for 6 h.[16] The same work-up gave a crude mixture of products as a yellow oil. Column chromatography using hexane–ethyl acetate (20 : 1 v/v) gave the title compound (**19b**) (3.04 g, 81% from **12b**). Recrystallization from hexane gave pale yellow plates, mp 52—53 °C. *Anal.* Calcd for $C_{12}H_{13}NO$: C, 76.98; H, 7.00; N, 7.48. Found: C, 77.07; H, 7.08; N, 7.45. IR ν_{max} cm⁻¹: 1660 (CO). ¹H-NMR δ: 1.20 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 2.94 (2H, q, J=7.5 Hz, $C\underline{H}_2CH_3$), 3.99 (3H, s, NCH_3), 6.9—7.4 (4H, m, Ar-H), 7.5—7.7 (1H, m, C_4-H). MS m/z: 187 (M^+, 65%), 158 (BP).

Further elution with the same solvent after obtaining **19b** gave 1-(1-methyl-1*H*-indol-3-yl)-1-propanone (70 mg, 1.9% from **12b**). Recrystallization from hexane gave pale yellow plates, mp 82—83 °C. *Anal.* Calcd for $C_{12}H_{13}NO$: C, 76.98; H, 7.00; N, 7.48. Found: C, 77.02; H, 7.00; N, 7.52. IR ν_{max} cm⁻¹: 1640 (C=O). ¹H-NMR δ: 1.25 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.83 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 3.75 (3H, s, NCH_3), 7.1—7.4 (3H, m, Ar-H), 7.58 (1H, s, C_2-H), 8.1—8.5 (1H, m, C_5-H). MS m/z: 187 (M^+, 33%), 158 (BP).

1-(1*H*-Indol-2-yl)-1-propanone (19c) A solution of 1-(1-benzyl-1*H*-indol-2-yl)-1-propanone (**19a**) (2.640 g, 10.0 mmol) in benzene (30 ml) was added to anhydrous $AlCl_3$ (5.350 g, 40.1 mmol) in benzene (20 ml) under an argon atmosphere, and the mixture was stirred at room temperature for 20 min, then added to 5% aqueous $NaHCO_3$ (100 ml). The reaction mixture was stirred for 30 min, and extracted with methylene chloride. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residual oil (3.234 g) was column-chromatographed using benzene to give the title compound (**19c**) (1.661 g, 96%). Recrystallization from benzene gave colorless plates, mp 153—154.5 °C. *Anal.* Calcd for $C_{11}H_{11}NO$: C, 76.28; H, 6.40; N, 8.09. Found: C, 76.29; H, 6.38; N, 8.07. IR ν_{max} cm⁻¹: 3305 (NH), 1650 (C=O). ¹H-NMR δ: 1.29 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 3.00 (2H, q, J=7.5 Hz, $COC\underline{H}_2CH_3$), 7.0—7.9 (5H, m, Ar-H), 9.2—9.7 (1H, br s, NH). MS m/z: 173 (M^+, 50%), 144 (BP).

1-(1-Benzyl-1*H*-indol-2-yl)-1-propanone Oximes [(*E*)- and (*Z*)-20a] 1-(1-Benzyl-1*H*-indol-2-yl)-1-propanone (**19a**) (2.630 g, 10 mmol) was added to a solution of hydroxylamine hydrochloride (1.463 g, 21 mmol) and AcONa (1.666 g, 20 mmol) in water (10 ml) and ethanol (40 ml). The mixture was refluxed for 3 h, then poured into water extracted with benzene, and dried over $MgSO_4$. Removal of the solvent *in vacuo* gave a yellow oil (3.01 g) which was separated into two oximes, (*E*)- or (*Z*)-**20a** (1.415 g, 51%) and (*Z*)- or (*E*)-**20a** (0.345 g, 12%), by column chromatography using hexane–ethyl acetate (10 : 1 v/v).

The first oxime [(*E*)- or (*Z*)-**20a**] was recrystallized from benzene–hexane to give colorless prisms, mp 129—131 °C. *Anal.* Calcd for $C_{18}H_{18}N_2O$: C, 77.67; H, 6.52; N, 10.06. Found: C, 77.89; H, 6.55; N, 9.96. IR ν_{max} cm⁻¹: 3300 (OH). ¹H-NMR δ: 1.08 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.75 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 5.68 (2H, s, $NC\underline{H}_2Ph$), 6.7—7.4 (9H, m, Ar-H and NOH), 6.80 (1H, s, C_3-H), 7.4—7.7 (1H, m, C_4-H). MS m/z: 278 (M^+, 44%), 91 (BP).

The second oxime [(*Z*)- or (*E*)-**20a**] was recrystallized from benzene–hexane to give colorless prisms, mp 165—166.5 °C. *Anal.* Calcd for $C_{18}H_{18}N_2O$: C, 77.67; H, 6.52; N, 10.06. Found: C, 77.70; H, 6.57; N, 10.06. IR ν_{max} cm⁻¹: 3250 (OH). ¹H-NMR δ: 0.91 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.36 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 5.30 (2H, s, $NC\underline{H}_2Ph$), 6.51 (1H, s, C_3-H), 6.8—7.4 (8H, m, Ar-H), 7.50—7.75 (1H, m, C_4-H), 9.16 (1H, s, NOH). MS m/z: 278 (M^+, 44%), 261 and 91 (BP).

A separate experiment gave the oximes [(*E*)- and (*Z*)-**20a**] as a mixture in 83% yield.

1-(1-Methyl-1*H*-indol-2-yl)-1-propanone Oximes [(*E*)- and (*Z*)-20b] 1-(1-Methyl-1*H*-indol-2-yl)-1-propanone (**19b**) (936 mg, 5 mmol) was treated with hydroxylamine hydrochloride (702 mg, 10 mmol) in the same manner as described for the reaction of the benzyl compound (**19a**). The same work-up procedure gave the two oximes, (*E*)- or (*Z*)-**20b** (787 mg, 78%) and (*Z*)- or (*E*)-**20b** (114 mg, 11%).

The first oxime [(*E*)- or (*Z*)-**20b**] was recrystallized from benzene–hexane to give colorless prisms, mp 147—149.5 °C. *Anal.* Calcd for $C_{12}H_{14}N_2O$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.47; H, 6.97; N, 13.63. IR ν_{max} cm⁻¹: 3250 (OH), 1665 (C=N). ¹H-NMR δ: 1.21 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.82 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 3.88 (3H, s, NCH_3), 6.70 (1H, s, C_3-H), 6.9—7.9 (5H, m, Ar-H and NOH). MS m/z: 202 (M^+, 98%), 130 (BP).

The second oxime [(*Z*)- or (*E*)-**20b**] was recrystallized from benzene–hexane to give colorless prisms, mp 158—161 °C. *Anal.* Calcd for $C_{12}H_{14}N_2O$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.46; H, 6.98; N, 13.70. IR ν_{max} cm⁻¹: 3200 (OH), 1645 (C=N). ¹H-NMR δ: 1.10 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 2.59 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 3.61 (3H, s, N-CH_3), 6.41 (1H, s, C_3-H), 6.9—7.7 (4H, m, Ar-H), 8.9 (1H, br s, NOH). MS m/z: 202 (M^+, 96%), 130 (BP).

1-(1*H*-Indol-2-yl)-1-propanone Oxime (20c) 1-(1*H*-Indol-2-yl)-1-propanone (**19c**) (341 mg, 1.97 mmol) was treated with hydroxylamine hydrochloride (292 mg, 4 mmol) in the same manner as described for the reaction of the benzyl compound (**19a**). The same work-up procedure gave a mixture of two oximes (348 mg, 94%). As it was hard to separate the mixture, the oxime was characterized as the (*E*)- and (*Z*)-mixture.

Recrystallization from benzene gave colorless prisms, mp 102.5—111.5 °C. *Anal.* Calcd for $C_{11}H_{12}N_2O$: C, 70.19; H, 6.43; N, 14.88. Found: C, 70.16; H, 6.38; N, 14.53. IR ν_{max} cm⁻¹: 3425 (NH), 1610 (C=N). ¹H-NMR δ: 1.27 and 1.31 (totally 3H, t, J=8.0 Hz, $CH_2C\underline{H}_3$), 2.79 and 2.81 (totally 2H, q, J=8.0 Hz, $C\underline{H}_2CH_3$), 6.74 and 6.77 (totally 1H, s, C_3-H), 6.9—7.8 (totally 4H, m, Ar-H), 8.5 and 8.9 (totally 2H, br s, NH and OH). MS m/z: 188 (M^+, BP).

***N*-[1-(1-Benzyl-1*H*-indol-2-yl)propyl]formamide (21a)** a) From the Oxime (**20a**): A solution of 1-(1-benzyl-1*H*-indol-2-yl)-1-propanone oxime [**20a**, a mixture of (*E*)- and (*Z*)-form] (278 mg, 1.0 mmol) in 1,2-dimethoxyethane (2 ml) was added to a solution of $NaBH_4$ (158 mg, 4.2 mmol) and $TiCl_4$[17] (0.23 ml, 2.1 mmol) in 1,2-dimethoxyethane (3 ml) under an argon atmosphere. The mixture was stirred at 50—60 °C for 1.5 h, then the reaction was quenched by adding water (10 ml), and the whole was made alkaline with concentrated NH_4OH, and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residual yellow oil (254 mg) of the corresponding amine was treated with ethyl formate (2.5 ml), and the mixture was refluxed for 2 h. After the reaction was complete, the excess ethyl formate was evaporated off *in vacuo* to leave a pale orange oil (254 mg). The oil was purified by column chromatography using hexane–ethyl acetate (2 : 1) to give the title compound (**21a**) as a solid (122 mg, 42%). Recrystallization from benzene gave colorless prisms, mp 148.5—150.5 °C. *Anal.* Calcd for $C_{19}H_{20}N_2O$: C, 78.05; H, 6.89; N, 9.58. Found: C, 78.31; H, 6.93; N, 9.30. IR ν_{max} cm⁻¹: 3300 (NH), 1650 (C=O). ¹H-NMR δ: 0.87 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.6—2.2 (2H, m, $CHC\underline{H}_2CH_3$), 4.9—5.7 (2H, m, $C\underline{H}NH$ and NH), 5.32 (2H, s, $NC\underline{H}_2Ph$), 6.45 (1H, s,

C_3-H), 6.7—7.4 (8H, m, Ar-H), 7.4—7.8 (1H, m, C_4-H), 7.80 (1H, s, CHO). MS *m/z*: 292 (M^+, 48%), 91 (BP).

b) By Leuckart Reaction: 1-(1-Benzyl-1*H*-indol-2-yl)-1-propanone (**19a**) (3.00 g, 11 mmol) was mixed with formic acid (10 ml), formamide (20 ml), and $(NH_4)_2SO_4$ (600 mg) in an autoclave. The mixture was stirred at 190 °C for 7 h, then poured into water and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (3.0 g) was purified by column chromatography using ethyl acetate–hexane (1 : 4) to give the title compound (**21a**) (1.82 g, 55%). Recrystallization from benzene gave colorless needles, mp 149—150.5 °C. *Anal.* Calcd for $C_{19}H_{20}N_2O$: C, 78.05; H, 6.89; N, 9.58. Found: C, 77.65; H, 6.86; N, 9.33.

***N*-[1-(1-Methyl-1*H*-indol-2-yl)propyl]formamide (21b)** 1-(1-Methyl-1*H*-indol-2-yl)-1-propanone oxime [**20b**, a mixture of (*E*)- and (*Z*)-form] (1.013 g, 5 mmol) was treated with $NaBH_4$ (764 mg, 20 mmol) and $TiCl_4$ (1.1 ml, 10 mmol) in 1,2-dimethoxyethane in the same way as described for the reaction of the benzyl compound (**20a**). Subsequent treatment of the corresponding amine (752 mg) with ethyl formate (12.5 ml) gave the title compound (**21b**) (550 mg, 51% from **21b**). Recrystallization from benzene gave colorless prisms, mp 128—130.5 °C. *Anal.* Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.21; H, 7.46; N, 12.66. IR ν_{max} cm^{-1}: 3240 (NH), 1675, 1650 (C=O). ^{1}H-NMR δ: 1.01 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.7—2.2 (2H, m, $CHC\underline{H}_2CH_3$), 3.61 (3H, s, NCH_3), 5.0—5.5 (1H, m, $C\underline{H}NHCHO$), 5.6—6.0 (1H, m, NH), 6.33 (1H, s, C_3-H), 6.8—7.6 (4H, m, Ar-H), 8.01 (1H, s, CHO). MS *m/z*: 216 (M^+, 81%), 187 (BP).

***N*-[1-(1*H*-Indol-2-yl)propyl]formamide (21c)** 1-(1*H*-Indol-2-yl)-1-propanone oxime (**20c**) (380 mg, 2.0 mmol) was treated with $NaBH_4$ (432 mg, 11.4 mmol) and $TiCl_4$ (0.92 ml, 8.4 mmol) in 1,2-dimethoxyethane in the same way as described for the reaction of the benzyl compound (**20a**). Subsequent treatment of the corresponding amine (277 mg) with ethyl formate (12 ml) gave the title compound (**21c**) (197 mg, 48% from **20c**) as a solid. Recrystallization from benzene–hexane gave colorless prisms, mp 112—114 °C. *Anal.* Calcd for $C_{12}H_{14}N_2O$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.52; H, 7.00; N, 13.70. IR ν_{max} cm^{-1}: 3350, 3300 (NH), 1640 (C=O). ^{1}H-NMR δ: 0.99 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.7—2.3 (2H, m, $CHC\underline{H}_2CH_3$), 4.8—5.3 (1H, m, $CH_2C\underline{H}NH$), 5.8—6.2 (1H, br, NH), 6.29 (1H, br s, C_3-H), 6.9—7.7 (4H, m, Ar-H), 8.1 (1H, br s, CHO), 8.8—9.3 (1H, br, NH). MS *m/z*: 202 (M^+, 97%), 173 and 118 (BP).

Ethyl *N*-[1-(1-Benzyl-1*H*-indol-2-yl)propyl]-*N*-formylaminoacetate (22a) A solution of *N*-[1-(1-benzyl-1*H*-indol-2-yl)propyl]formamide (**21a**) (500 mg, 1.7 mmol) in dimethyl sulfoxide (DMSO) (4 ml) was added slowly to a suspension of 60% NaH (84 mg, 2.1 mmol) under an argon atmosphere. The mixture was stirred at 50 °C for 3 h. To this solution, ethyl chloroacetate (0.552 ml, 5.2 mmol) was added. The reaction mixture was stirred at 90 °C for 4 h, poured into ice-water and extracted with ether. The organic layer was washed with saturated aqueous NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was column-chromatographed using hexane–ethyl acetate (4 : 1) to give the title compound (**22a**) (450 mg, 70%) after recovery of the starting material (**21a**) (140 mg, 28%). Recrystallization of **22a** from benzene gave colorless prisms, mp 114.5—116 °C. *Anal.* Calcd for $C_{23}H_{26}N_2O_3$: C, 72.99; H, 6.92; N, 7.40. Found: C, 72.71; H, 7.00; N, 7.38. IR ν_{max} cm^{-1}: 1735; 1680 (C=O). ^{1}H-NMR (DMSO-d_6, 100 MHz) δ: 0.71 and 0.88 (totally 6H, each t, J=7.5 Hz, $2\times CH_2C\underline{H}_3$), 1.7—2.1 (2H, m, $CHC\underline{H}_2CH_3$), 3.3—4.0 (4H, m, $OC\underline{H}_2CH_3$ and $COCH_2N$), 4.7—5.1 (1H, m, $NC\underline{H}CH_2$), 5.1—5.6 (2H, m, NCH_2Ph), 6.60 and 6.65 (totally 1H, each s, C_3-H), 6.8—7.3 (8H, m, Ar-H), 7.3—7.6 (1H, m, C_4-H), 8.03 and 8.25 (totally 1H, each s, CHO). MS *m/z*: 378 (M^+, BP).

Ethyl *N*-Formyl-*N*-[1-(1-methyl-1*H*-indol-2-yl)propyl]aminoacetate (22b) *N*-[1-(1-Methyl-1*H*-indol-2-yl)propyl]formamide (**21b**) (216 mg, 1.0 mmol) was treated with 50% NaH (72 mg, 1.5 mmol) and ethyl chloroacetate (0.324 ml, 3 mmol) in the same manner as described for the reaction of the corresponding benzyl compound (**21a**). After work-up, the glycinate (**22b**) (199 mg, 66%) was obtained as a pale yellow oil, with recovery of the starting material (**21b**) (40 mg, 19%). IR ν_{max} cm^{-1}: 1740, 1660 (C=O). ^{1}H-NMR δ: 0.6—1.5 (6H, m, $2\times CH_2C\underline{H}_3$), 1.7—2.3 (2H, m, $CHC\underline{H}_2CH_3$), 3.4—4.4 (7H, m, $OC\underline{H}_2CH_3$, $NC\underline{H}_2CO$, NCH_3), 4.68 and 5.71 (totally 1H, each dif t, J=7.5 Hz, $NC\underline{H}CH_2$), 6.44 and 6.48 (toally 1H, each s, C_3-H), 6.9—7.7 (4H, m, Ar-H), 8.13 and 8.34 (totally 1H, each s, CHO). MS *m/z*: 302 (M^+, BP). High-resolution MS: Calcd for $C_{17}H_{22}N_2O_3$: 302.1625. Found: 302.1644.

***N*-{1-[1-(Ethoxycarbonylmethyl)-1*H*-indol-2-yl]propyl}formamide (23)** A solution of *N*-[1-(1*H*-indol-2-yl)propyl]formamide (**21c**) (79 mg, 0.39 mmol) in dimethylformamide (DMF) (2 ml) was added to a suspension of 60% NaH (24 mg, 0.6 mmol) in DMF (1 ml) under an argon atmosphere. The mixture was stirred at 50 °C for 3.5 h, and, after cooling, ethyl bromoacetate (0.135 ml, 1.2 mmol) was added. The mixture was stirred at room temperature for 50 min and at 50 °C for 30 min, poured into ice-water and extracted with ether. The organic layer was washed with saturated aqueous NaCl, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (134 mg) was column-chromatographed using hexane–ethyl acetate (3 : 1 v/v) to give the title compound (**23**) (22 mg, 20%) and the starting material (**21c**) (32 mg, 40%). Recrystallization of **23** from benzene–hexane gave pale yellow prisms, mp 131—135 °C. IR ν_{max} cm^{-1}: 3280 (NH), 1740, 1640 (C=O). ^{1}H-NMR δ: 1.03 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.26 (3H, t, J=7.0 Hz, $OCH_2C\underline{H}_3$), 1.6—2.4 (2H, m, $CHC\underline{H}_2CH_3$), 4.16 (2H, q, J=7.0 Hz, $OC\underline{H}_2CH_3$), 4.88 (2H, s, $NC\underline{H}_2CO$), 5.1—5.4 (1H, m, $NC\underline{H}CH_2$), 5.5—6.0 (1H, br, NH), 6.45 (1H, s, C_3-H), 6.9—7.7 (4H, m, Ar-H), 8.01 (1H, dif s, CHO). MS *m/z*: 288 (M^+, 96%), 259 (BP). High-resolution MS: Calcd for $C_{16}H_{20}N_2O_3$: 288.1475. Found: 288.1435.

9-Benzyl-1-ethyl-2-formyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (24a) A mixture of ethyl *N*-[1-(1-benzyl-1*H*-indol-2-yl)propyl]-*N*-formylaminoacetate (**22a**) (300 mg, 0.79 mmol) and PPA (10 g) was stirred at 70 °C for 5 h. After cooling, the reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue (326 mg) was separated into each component by column chromatography using hexane–ethyl acetate (2 : 1), the starting material (**22a**) (32 mg, 11%) and the title compound (**24a**) (210 mg, 80%). Recrystallization of **24a** from methanol gave colorless prisms, mp 107—113 °C. *Anal.* Calcd for $C_{21}H_{20}N_2O_2$: C, 75;88; H, 6.06; N, 8.43. Found: C, 75.86; H, 6.08; N, 8.50. IR ν_{max} cm^{-1}: 1660, 1640 (C=O). ^{1}H-NMR δ: 1.00 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.5—2.1 (2H, m, $CHC\underline{H}_2CH_3$), 4.18 (2H, s, C_3-H), 5.40 (2H, s, $C\underline{H}_2Ph$), 5.7—6.1 (1H, m, C_1-H), 6.9—7.5 (8H, m, Ar-H), 8.1—8.4 (1H, m, C_5-H), 8.23 (1H, s, CHO). MS *m/z*: 332 (M^+, 47%), 303 (BP).

1-Ethyl-2-formyl-9-methyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (24b) A mixture of ethyl *N*-formyl-*N*-[1-(1-methyl-1*H*-indol-2-yl)propyl]aminoacetate (**22b**) (330 mg, 1.09 mmol) and PPA (4.0 g) was stirred at 75 °C for 40 min. The same work-up procedure as described for the reaction of the benzyl compound (**22a**) gave the title compound (**24b**) (214 mg, 76%). Recrystallization from ethyl acetate gave colorless needles, mp 180—181.5 °C. *Anal.* Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.21; H, 6.32; N, 10.96. IR ν_{max} cm^{-1}: 1675, 1665, 1650 (C=O). ^{1}H-NMR δ: 1.18 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.7—2.2 (2H, m, $CHC\underline{H}_2CH_3$), 3.81 (3H, s, NCH_3), 4.20 (2H, s, C_3-H), 5.88 (1H, t, J=8.0 Hz, C_1-H), 7.2—7.5 (3H, m, Ar-H), 8.1—8.4 (1H, m, C_5-H), 8.25 (1H, s, CHO). MS *m/z*: 256 (M^+, 40%), 227 (BP).

9-Benzyl-1-ethyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (25a) Concentrated HCl (0.5 ml) was added to a solution of 9-benzyl-1-ethyl-2-formyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (**24a**) (460 mg, 1.38 mmol) in methanol (2.5 ml). The reaction mixture was stirred at 60 °C for 2.5 h, poured into water, made alkaline with saturated aqueous $NaHCO_3$, and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$, and evaporated to dryness *in vacuo* to give pale yellow prisms (403 mg, 96%), mp 156—160 °C. Recrystallization from methanol gave colorless needles, mp 165.5—167.5 °C. *Anal.* Calcd for $C_{20}H_{20}N_2O$: C, 78.92; H, 6.62; N, 9.20. Found: C, 78.86; H, 6.69; N, 9.17. IR ν_{max} cm^{-1}: 3335 (NH), 1655 (C=O). ^{1}H-NMR δ: 1.10 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.4—1.9 (2H, m, $CHC\underline{H}_2CH_3$), 2.41 (1H, s, NH), 3.35 and 3.72 (each 1H, d, J=18.0 Hz, C_3-H), 3.96 (1H, dd, J=9.5 and 4.0 Hz, C_1-H), 5.27 (2H, s, $C\underline{H}_2Ph$), 6.9—7.5 (8H, m, Ar-H), 8.1—8.4 (1H, m, C_5-H). MS *m/z*: 304 (M^+, 7%), 91 (BP).

1-Ethyl-9-methyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (25b) Concentrated HCl (0.14 ml) was added to a solution of 1-ethyl-2-formyl-9-methyl-4-oxo-1,2,3,4-tetrahydro-*β*-carboline (**24b**) (100 mg, 0.39 mmol) in methanol (1.0 ml), and the mixture was stirred at 60 °C for 3.5 h. The same work-up procedure as described for the reaction of the benzyl compound (**24b**) gave the title compound (**25b**) as pale brown crystals (87 mg, 98%), mp 179—190 °C. Recrystallization from ethyl acetate gave colorless prisms, mp 198—200 °C. *Anal.* Calcd for $C_{14}H_{16}N_2O$: C, 73.66; H, 7.06; N, 12.27. Found: C, 73.66; H, 7.10; N, 12.21. IR ν_{max} cm^{-1}: 3305 (NH), 1635 (C=O). ^{1}H-NMR δ: 1.20 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 1.5—2.1 (2H, m, $CHC\underline{H}_2CH_3$), 2.38 (1H, s, NH), 3.32 and 3.70 (each 1H, d, J=17.0 Hz, C_3-H), 3.60 (3H, s, NCH_3), 3.8—4.2 (1H, m, $C\underline{H}CH_2CH_3$), 7.2—7.5 (3H, m, Ar-H), 8.1—8.4 (1H, m, C_5-H). MS *m/z*: 228 (M^+, 13%), 199 (BP).

9-Benzyl-1-ethyl-4-hydroxy-β-carboline (26a) A 10% Pd–C catalyst (130 mg) was added to a solution of 9-benzyl-1-ethyl-4-oxo-1,2,3,4-tetrahydro-β-carboline (**25b**) (380 mg, 1.25 mmol) in decalin (3 ml), and the mixture was stirred at 140 °C for 8 h. After the reaction was complete, the mixture was filtered under suction, and the residue was washed with ethyl acetate. The combined filtrate and washings were evaporated to dryness *in vacuo*. The residue (241 mg, 64%) was recrystallized from methanol to give pale brown prisms, mp 230—240 °C. IR ν_{max} cm^{-1}: *ca.* 2500 (br, OH). ^{1}H-NMR (DMSO-d_6) δ: 1.20 (3H, t, J=7.5 Hz, $CH_2C\underline{H}_3$), 3.03 (2H, q, J=7.5 Hz, $CHC\underline{H}_2CH_3$), 5.84 (2H, s, $C\underline{H}_2Ph$), 6.8—7.7 (8H, m, Ar-H), 7.97 (1H, s, C_3-H), 8.38 (1H, d, J=8.0 Hz, C_5-H), 10.1 (1H, br s, OH). MS *m/z*: 302 (M$^+$, 51%), 91 (BP). High-resolution MS: Calcd for $C_{20}H_{18}N_2O$: 302.1420. Found: 302.1435.

1-Ethyl-4-hydroxy-9-methyl-β-carboline(Isocrenatine) (26b) A mixture of 1-ethyl-9-methyl-4-oxo-1,2,3,4-tetrahydro-β-carboline (**25b**) (105 mg, 0.46 mmol) and 10% Pd–C (35 mg) in decalin (2 ml) was stirred at 140 °C for 4 h and at 180 °C for 2 h. After cooling, the reaction mixture was directly column-chromatographed using methylene chloride–methanol (20:1 v/v) to give pale brown crystals (61 mg, 59%), mp 260—270 °C (dec.). Recrystallization from methanol gave pale brown prisms, mp 276—282 °C (dec.). *Anal.* Calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24; N, 12.38. Found: C, 74.13; H, 6.32; N, 12.24. IR ν_{max}^{KBr} cm^{-1}: 3600—3300 (OH). ^{1}H-NMR (400 MHz) δ: shown in Table II. MS *m/z*: 226 (M$^+$, BP).

9-Benzyl-1-ethyl-4-methoxy-β-carboline (27) Powdered K_2CO_3 (237 mg, 1.71 mmol) and dimethyl sulfate (0.132 ml, 1.40 mmol) were added to a solution of 9-benzyl-1-ethyl-4-hydroxy-β-carboline (**26a**) (100 mg, 0.33 mmol) in methanol (2.5 ml), and the mixture was stirred at 64 °C for 10 min. After cooling, the reaction mixture was made alkaline with concentrated NH_4OH and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was column-chromatographed using hexane–ethyl acetate (2:1 v/v) to give the title compound (**27**) as a solid (41 mg, 39%). Recrystallization from methanol gave colorless prisms, mp 107—115 °C. IR: no characteristic band. ^{1}H-NMR (90 MHz) δ: 1.31 (3H, t, J=7.0 Hz, $CH_2C\underline{H}_3$), 3.08 (2H, q, J=7.0 Hz, $C\underline{H}_2CH_3$), 4.14 (3H, s, OCH_3), 5.73 (2H, s, $C\underline{H}_2Ph$), 6.8—7.5 (8H, m, Ar-H), 8.02 (1H, s, C_3-H), 8.37 (1H, d, J=8.0 Hz, C_5-H). MS *m/z*: 316 (M$^+$, BP). High-resolution MS: Calcd for $C_{21}H_{20}N_2O$: 316.1577. Found: 316.1602.

The 4-hydroxy compound (**26a**) was methylated to **27** with diazomethane in a mixed solvent of ethyl acetate and ether in 30% yield.

1-Ethyl-4-methoxy-β-carboline (Crenatine) (1a) A solution of 9-benzyl-1-ethyl-4-methoxy-β-carboline (**27**) (26 mg, 0.082 mmol) in anisole (2 ml) was added to $AlCl_3$ (250 mg, 1.87 mmol) under ice-cooling in an argon atmosphere. The reaction mixture was stirred at room temperature for 23 h, poured into ice-water and extracted with ethyl acetate. The organic layer was washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. Removal of the solvent *in vacuo* left the residue, which was purified by column-chromatography using hexane–ethyl acetate (1:1 v/v) to give crenatine as crystals (13 mg, 70%). Recrystallization from ethanol gave colorless leaflets, mp 180—182 °C. *Anal.* Calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24; N, 12.38. Found: C, 74.34; H, 6.32; N, 12.38. IR ν_{max}^{KBr} cm^{-1}: 3420 (br, NH). ^{1}H-NMR (90 MHz): shown in Table II. MS *m/z*: 226 (M$^+$, BP). High resolution MS: Calcd for $C_{14}H_{14}N_2O$: 226.1107. Found: 226.1103.

The synthetic crenatine was identical with the natural product[3a)] in all respects including mixed melting point experiment.

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Photosensitized Oxidation of Isoeugenol in Protic and Aprotic Solvents

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Sensitized photooxygenation of isoeugenol gave seven products in methanol, seven products in ethanol, six products in acetone, and five products in acetonitrile. One of the products is a 7,7′-linked lignan of a type which has not yet been observed in nature. The structures of these products were elucidated and the mechanisms of their formation are discussed.

Keywords photooxidation; isoeugenol; lignan with 5–8′-, 8–O–4′-or 7–7′-linkage; biomimetic study; mechanism

The oxidation of isoeugenol (**1**) has been studied previously as a model of the formation of lignan-related dimers during ferric chloride oxidation[1] and enzymatic oxidation.[2] Photolysis[3] and free radical oxidation[4] of isoeugenol also give similar products. Further studies showed that free radical oxidation of isoeugenol[5] produced four trilignols. The anodic oxidation of isoeugenol gave dimerization products of a different type.[6] Few studies have been reported on the observation of the formation of lignan-related dimers in sensitized photooxidation. In connection with our interest in lignan and biomimetic studies, we recently studied sensitized photooxidation of methyl (*E*)-ferulate.[7] Sensitized photooxidation of isoeugenol (**1**) in methanol[8] (MeOH) had been reported to give dehydrodiisoeugenol (**2**) and **3a** (*erythro*+*threo*) (purified by acetylation and hydrogenation). The paper did not discuss the formation mechanism. In a previous communication, we described the result of photooxidation of isoeugenol in acetone solution.[9] In this paper, we present in detail the results of photooxidation of isoeugenol in alcohols, acetone, and acetonitrile. A solution of isoeugenol (**1**) and methylene blue in a solvent was irradiated using a fluorescent lamp. Seven products (**4a**, **3a**, **5a**, **2**, **6**, **7a**, and **8a**) in MeOH, seven products (**4b**, **3b**, **5b**, **2**, **6**, **7b**, and **8a**) in ethanol (EtOH), six products (**2**, **6**, **9**, **10a**, **11a**, and **8a**) in acetone, and five products (**2**, **6**, **10a**, **12a**, and **8a**) in acetonitrile, listed in the order of elution upon chromatography, were isolated from the reaction mixture after purification of silica gel. Compound **2** is the major product, and **6** is the next most major product. Products **2**, **3** and **8** represent three different linkages of lignans (5–8′, 4–O–7′; 8–O–4′; 7–7′, 8–O–O–8′). The presence of three differently linked lignans in one species of plant is very rare. Products **8a** and **9** are stable endoperoxides. The structures of all the products were elucidated as follows.

Compounds **3a** and **3b** were purified by acetylation and hydrogenation, so their original structures would have been **12b** and **12c**, respectively. Meanwhile compounds **5a**, **7a**,

1 (*trans*+*cis*)

2

3a : R_1=Ac, R_2=Me (*erythro*+*threo*)
3b : R_1=Ac, R_2=Et (*erythro*+*threo*)
3c : R_1=H, R_2=Me (*erythro*+*threo*)

4a : R=Me
4b : R=Et

threo form
5a : R_1=Ac, R_2=Me, R_3=H
5b : R_1=R_3=H, R_2=Et
5c : R_1=R_3=H, R_2=Me
5d : R_1=R_3=Ac, R_2=Et

erythro form
7a : R_1=R_3=Ac, R_2=Me
7b : R_1=R_3=Ac, R_2=Et
7c : R_1=R_3=H, R_2=Me
7d : R_1=R_3=H, R_2=Et

6

Table I. ^{1}H-NMR Data for **5a**, **5b**, **5d**, **7a**, **7b**, and **13**

H	5a	5b	5d	7a	7b	13
2, 5, 6	6.89—7.25 ABX system	6.69—6.88 ABX system	6.89—7.03 ABX system	6.82—7.05 ABX system	6.88—7.10 ABX system	6.31—6.94 ABX system
7	4.08 d (4.2)[a)]	3.98 d (5.0)	4.31 d (5.0)	4.30 d (4.4)	4.13 d (7.0)	3.04 d (7.0)
8	3.89 m	3.75 m	5.00 m	5.03 m	4.92 m	4.30 m
9	1.13 d (6.2)	1.10 d (6.9)	1.16 d (7.0)	1.20 d (7.0)	1.00 d (7.0)	1.04 d (6.2)
C$\underline{H}_3$CH$_2$O–		1.18 t (7.0)	1.23 t (7.0)		1.17 t (7.0)	
CH$_3$C$\underline{H}_2$O–		3.36 m	3.43 m		3.36 m	
C$\underline{H}_3$O–Ar	3.83 s	3.89 s	3.83 s	3.84 s	3.82 s	3.62 s
C$\underline{H}_3$O–R	3.30 s			3.32 s		
C$\underline{H}_3$COOR			1.97 s	2.00 s	1.97 s	
C$\underline{H}_3$COO–Ar	2.31 s		2.30 s	2.31 s	2.24 s	2.28 s

a) Figures in parenthesis are coupling constants in Hz.

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7b, and **12a** were isolated after acetylation, so their original structures would have been **5c**, **7c**, **7d**, and **12d**, respectively. Products **2** (mp 132—134 °C) and **6** (mp 80—82 °C) were identical with dehydrodiisoeugenol,[1,8] and vanillin, respectively. By comparison of the spectral data with reported values,[8] the structures of **3a** and **3b** were elucidated to be as shown. Chemical correlation between **3a** and **3b** was made as follows. When **3b** was treated with *p*-toluenesulfonic acid in MeOH solution, it gave **3c** which was identical with the product obtained from **3a** by basic hydrolysis. Compounds **4a** and **4b** were identical with methyl vanillate and ethyl vanillate, respectively. Compounds **5a**, **5b**, **7a**, **7b**, **8a**, and **9** give a positive KI test in acidic acetone solution. The results show that these compounds are peroxides. Compounds **5a** and **7a** showed similar proton nuclear magnetic resonance (^{1}H-NMR) spectra (Table I), except for an additional acetyl group in **7a**. Further, the H-8 proton exhibits different chemical shifts: δ 5.03 (1H, m) in **7a** and δ 3.89 (1H, m) in **5a**. Chemical correlation between **5a** and **7a** was achieved on catalytic hydrogenolysis with Pd–C as the catalyst in MeOH, compounds **5a** and **7a** gave the same product (**13**), which shows infrared (IR) absorption bands at 3600 (–OH), 1750 (Ar-OAc) cm^{-1}. Dehydration of **13** in acetone with *p*-toluenesulfonic acid under reflux afforded *trans*-isoeugenol (**1**). From the foregoing results, **5c** and **7c** are diastereomers. The *threo* form (**5**) favors hydrogen bonding (as in formula **14**), rather than the *erythro* form. Compounds **5b** and **5c** were eluted with less polar eluent (5% ethyl acetate in hexane) and **7c** and **7d** with more polar eluent (20% ethyl acetate in hexane). Therefore, we can assign **5** as *threo* form and **7** as *erythro* form, because **5** with greater hydrogen bonding ability to hydroxyl groups is less well adsorbed by silica gel and is eluted more easily by a less polar solvent. Compound **5a** resisted acetylation with Ac_2O/pyridine at room temperature overnight. The result also indicates that **5a** is a *threo* form. Acetylation of **5b** with Ac_2O/pyridine at 60 °C afforded **5d** which showed similar ^{1}H-NMR signals to **7b**. The assignment of the structures of **5b** and **7b** was based on their physical data. Meanwhile **5a**, **5d**, **7a**, and **7b** gave the same product **13** on catalytic hydrogenolysis with Pd–C as the catalyst. Compound **9**, mp 74—76 °C (from MeOH), is an endoperoxide, giving a positive KI test in acidic acetone solution and no hydroxyl absorption band in its IR spectrum. The ultraviolet (UV) spectrum (λ_{max} 209, 250, 275 nm) and IR spectrum (ν_{max} 1690, 1650, 1610 cm^{-1}) suggested the presence of a dienone moiety with a methoxyl group at the α-position. The ^{1}H-NMR data confirm the structure to be as shown. Compound **10a**, mp 73—75 °C (from $CHCl_3$), an aldehyde, exhibits IR absorption bands at 3470, 3050, 1660, 1110 cm^{-1} and ^{1}H-NMR signals at δ 1.41 (3H, d, J=7.0 Hz), 3.51 (1H, m), 3.88 and 3.92 (each 3H, s, –OMe), 5.23 (1H, d, J=10 Hz), 6.24 (1H, br s, –OH), 6.82—7.38 (5H, m), 9.82 (1H, s). Compound **10a**, on reaction with Ac_2O in pyridine, gave an amorphous monoacetate (**10b**) [ν_{max} 3050, 1770, 1680 cm^{-1}; δ 2.28 (3H, s)]. Upon reduction with $NaBH_4$, **10a** afforded an alcohol (**15**) [mp 84—85 °C (from $CHCl_3$); ν_{max} 3390 cm^{-1}; δ 4.68 (2H, s)]. From the above data, the structure of **10a** is similar to that of dehydrodiisoeugenol (**2**) except for a formyl group instead of a propenyl group. It is proposed that **10a** was derived from **2** by photooxidation. Indeed **2** yielded **10a** upon sensitized photooxidation. Compound **11a**, mp 134—136 °C (from MeOH), contains a hydroxyl group (ν_{max} 3350 cm^{-1}), two methoxyl groups [δ 3.89 and 3.98 (each 3H, s)], one aldehyde [ν_{max} 1680 cm^{-1}; δ 9.96 (1H, s)], an ester group (ν_{max} 1720 cm^{-1}), and six phenyl protons [δ 6.96—7.88 (6H, m)]. The acetylation of **11a** with Ac_2O–pyridine at room temperature yielded a monoacetate (**11b**) [mp 158—160 °C; ν_{max} 1750 cm^{-1}; δ 2.34 (3H, s)], Methyl vanillate (**4a**) and vanillin (**6**) were obtained from **11a** by heating in acidic MeOH solution. Compound **8a** (mp 192—194 °C), a six membered ring endoperoxide, is stable to $NaBH_4$ reduction and gave a positive KI test under acidic conditions. The formula $C_{20}H_{24}O_6$ was derived from a mass measurement (mass spectra (MS) m/z=360) and elemental analysis. Compound **8a** shows ^{1}H-NMR signals at δ 1.01 (6H, d, J=6.0 Hz), 2.66 (2H, m, AA′, –C<u>H</u>Ar), 3.74 (6H, s, 2×–OMe), 4.51 (2H, m, XX′, –C<u>H</u>Me), 5.41 (2H, s, 2×–OH), 6.34 (2H, d, J=1.8 Hz), 6.59 (2H, dd, J=8.1, 1.8 Hz), and 6.86 (2H, d, J=8.1 Hz). Irradiation of the methyl signal (δ 1.01) simplified the multiplet at δ 4.51

8a : R = 4-hydroxy-3-methoxyphenyl (OMe, OH)
8b : R = 4-acetoxy-3-methoxyphenyl (OMe, OAc)

9

10a : R=H
10b : R=Ac

11a : R=H
11b : R=Ac

12a : R_1=R_2=Ac (*erythro*+ *threo*)
12b : R_1=H, R_2=Me (*erythro*+ *threo*)
12c : R_1=H, R_2=Et (*erythro*+ *threo*)
12d : R_1=R_2=H (*erythro*+ *threo*)

13

14 (*threo*)

15

16

to a doublet ($J=8.0$ Hz). Irradiation of the multiplet at δ 4.51 caused the multiplet at δ 2.66 and the doublet at δ 1.01 to collapse to a singlet each. The signal at δ 4.51 became a quartet ($J=6.0$ Hz) upon irradiation of the multiplet at δ 2.66. According to the above evidence, **8a** is a symmetric compound, and the four substituents are all in equatorial orientation as shown in the formula. Its diacetate (**8b**) (ν_{max} 1770 cm^{-1}; no hydroxyl absorption band) gave a similar ^{1}H-NMR spectrum to **8a**, except for a signal at δ 2.25 (6H, s) instead of the signal at δ 5.41. Compound **12a**, a mixture of *erythro* and *threo* forms with 1 : 1 ratio, was structurally elucidated as shown from the spectra. Hydrogenation of **12a** (PtO_2 catalyst in ethyl acetate) and treatment with *p*-toluenesulfonic acid gave **3c** (*erythro* + *threo*), which was also obtained from **3a** by saponification.

Chart 1

Chart 2

The formation of the products by photosensitized oxidation of isoeugenol (**1**) may be rationalized in terms of the mechanisms depicted in Charts 1—3. As shown in Chart 1, the phenolic hydrogen of **1** is abstracted by $^1O_2$ or ^{3}sen*[10] to afford the RO· radical, which exhibits two other resonance hybrids, Rβ· and R5· radicals. Coupling of Rβ· and RO· radicals yields the 8–O–4′ type quinone intermediate (**17**), which subsequently adds ROH (R=H, Me or Et) to afford **12b**, **12c**, or **12d**. Combination of Rβ· and R5· radical mesomers produced the intermediate (**18**) that generates **2** *via* spontaneous cyclization. The reaction of singlet oxygen with a strained[11] or electron-rich[12] double bond gives the perepoxide. Dehydrodiisoeugenol (**2**) yields the perepoxide (**19**) by the addition of $^1O_2$ and is then transformed to the dioxetane (**20**),[13] which is cleaved (*via* the diradical or through concerted cleavage based on solvent)[14] to afford **10a**. The formation of **4a**, **4b**, **5b**, **5c**, **7c** or **7d** from isoeugenol (**1**) is *via* the perepoxide (**21**) (see Chart 2) obtained by the addition of $^1O_2$ to **1**. The perepoxide **21** rearranges into two species, the zwitterion (**22**) and the dioxetane (**23**). The addition of MeOH of EtOH to the zwitterion (**22**) would yield **5b**, **5c**, **7c** or **7d**. The product vanillin (**6**) would be obtained from the cleavage of the dioxetane (**23**). The formation of **4a** or **4b** presumably involves cleavage of the hydroperoxide (**25**) derived from the MeOH acetal (**24**) by oxidation with triplet oxygen. The acetal (**24**) would be derived from **6** during the sensitized photooxidation. With methylene blue as the sensitizer the acidity of the solution is increased, and it has been reported that aldehyde is converted to acetal in MeOH solution under such photooxidation conditions.[15] We[16] have reported that the sensitized photooxidation conditions catalyze the coupling of formaldehyde and benzamide (or acetamide) and convert maleic aldehydeacid and fumaric aldehydeacid to their corresponding pseudoesters. In order to prove the proposed mechanism, the following reaction was performed. When **6** was exposed to $^3O_2$ in alcohol solution (MeOH or EtOH) in the dark, no product was observed. But **6** can be oxidized with $^1O_2$ in alcohols (MeOH or EtOH) to produce **4a** or **4b**. Meanwhile, **4a** or **4b** can be prepared by oxidation with $^3O_2$ in alcohols with *p*-toluenesulfonic acid as a catalyst in the dark. Compounds **11a**, **9**, and **8a** derived from **6**, **1**, **21**, respectively, are shown in Chart 3. Abstraction of the phenolic hydrogen from vanillin (**6**) by $^1O_2$ or ^{3}sens* yields the radical (**26**), which adds to the aldehyde of **6** to produce

Chart 3

another radical (**27**). The generation of **11a** was achieved by transfer of hydrogen from **27** to **26**. The oxidation of vanillin (**6**) with $^1O_2$ in acetone solution was performed but it gave the ester **11a** in low yield. The [2+4] reaction is usually found in photooxidation of a styrene-type olefin and the thermally stable endoperoxide is formed stereospecifically.[17] The reaction of $^1O_2$ with isoeugenol (**1**) produced the enol (**28**) by [2+4] reaction, and then **28** tautomerized to the stable ketone (**9**). The formation of the endoperoxide (**8a**) is unique, resulting from cyclization between **21** and **1**. The other route for the formation of (**8a**) may be *via* the biradical (**16**), which may be formed by the addition of $^3O_2$ to two molecules of **1**, but this pathway can be excluded because oxidation of **1** with $^3O_2$ in the dark did not give **8a**, which is an unnatural lignan. No 7,7′-linked lignan has been found in nature.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer model 137 spectrometer. ^{1}H-NMR spectra were run on a JEOL TNM-FX-100 at 100 MHz with tetramethylsilane as an internal standard. Chemical shifts are given in δ values and coupling constants (J) are given in hertz (Hz). Electron impact mass spectra (EIMS) were taken on a Hitachi RMS-4.

Photooxidation of 1 in Alcohols, Acetone and Acetonitrile A solution of **1** (6 g) and methylene blue (100 mg) in MeOH, EtOH, acetone or acetonitrile (100 ml) was irradiated with 3 × 20 W fluorescent lamp. During the irradiation, oxygen was bubbled through the solution, which was cooled to 10—15 °C. The reaction was completed within 5 d. After removal of the solvent *in vacuo*, the residue was subjected to chromatography on silica gel. Seven products, **4a** (10 mg), **3a** (45 mg), **5a** (20 mg), **2** (0.94 g), **6** (0.65 g), **7a** (25 mg), and **8a** (5 mg), listed in their order of elution, were isolated from the MeOH solution. Seven products [**4b** (80 mg), **3b** (40 mg), **5b** (30 mg), **2** (1.0 g), **6** (0.6 g), **7b** (30 mg), and **8a** (4 mg)], six products [**2** (2.5 g), **6** (0.8 g), **9** (17 mg), **10a** (15 mg), **11a** (80 mg), and **8a** (10 mg)], and five products [**2** (1.63 g), **6** (1.32 g), **10a** (60 mg), **12a** (15 mg), and **8a** (7 mg)] were purified from the EtOH, acetone, and acetonitrile solutions, respectively, under the same conditions. Compounds **3a** and **3b** were purified by acetylation (Ac_2O/pyridine, room temperature, overnight) and hydrogenation (in ethyl acetate using Adams catalyst). Compounds **5a**, **7a**, **7b**, and **12a** were isolated after acetylation (same conditions as above). Products **2** (mp 132—134 °C), **6a** (mp 80—82 °C), **4a** (mp 68—70 °C), and **4b** were identical with dehydrodiisoeugenol,[1,8] vanillin,[7] methyl vanillate,[18] and ethyl vanillate,[19] based on comparison of their physical data with reference values or data for authentic samples. The physical data of new products were as follows.

3b: Oil. IR ν_{max}^{neat} cm^{-1}: 3040, 1758, 1600, 1500, 1420, 1265, 1200, 1140, 1040, 920, 845. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=7.0 Hz), 1.34 (3H, d, J=6.5 Hz), 1.60 (2H, m, Ar-CH_2C<u>H</u>$_2$$CH_3$), 2.28 (3H, s), 2.49 (2H, t, J=7.0 Hz, ArC<u>H</u>$_2$$CH_2CH_3$), 3.48 (2H, q, J=6.5 Hz, –OC<u>H</u>$_2$$CH_3$), 3.75 and 3.79 (each 3H, s), 4.25—4.55 (2H, m, –C<u>H</u>ORC<u>H</u>O–), 6.63—7.02 (6H, m, phenyl protons). NMR data of the two epimers were almost identical, except for a methyl signal of one isomer at δ 1.23 (3H, t, J=7.0 Hz, C<u>H</u>$_3$$CH_3O$–). *Anal.* Calcd for $C_{24}H_{34}O_6$: C, 69.21; H, 7.74. Found: C, 69.41; H, 7.79.

5a: Oil. IR ν_{max}^{neat} cm^{-1}: 3400, 3040, 1760, 1580, 1510, 1180, 1105, 1040, 955, 870, 825, 755. *Anal.* Calcd for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71. Found: C, 57.90; H, 6.63.

5b: Oil. IR ν_{max}^{neat} cm^{-1}: 3450, 1600, 1500, 1280, 1220, 1088, 1040, 925, 822, 775. *Anal.* Calcd for $C_{12}H_{18}O_5$: C, 59.49; H, 7.49. Found: C, 59.30; H, 7.55.

7a: Oil. IR ν_{max}^{neat} cm^{-1}: 3045, 1750, 1720, 1600, 1500, 1250, 1200, 1150, 1025, 915, 820, 790, 750. *Anal.* Calcd for $C_{15}H_{20}O_7$: C, 57.68; H, 6.46. Found: C, 57.81; H, 6.53.

7b: Oil. IR ν_{max}^{neat} cm^{-1}: 3045, 1762, 1735, 1605, 1510, 1255, 1210, 1130, 1050, 925, 860, 800, 785. *Anal.* Calcd for $C_{16}H_{22}O_7$: C, 58.88; H, 6.80. Found: C, 58.70; H, 6.88.

8a: mp 192—194 °C. IR ν_{max}^{KBr} cm^{-1}: 3450, 1600, 1500, 1275, 1250, 1200, 1120, 1040, 870, 830, 780, 767. MS m/z (%); 360 (M^+, 25), 345 (17), 300 (21), 285 (25), 274 (32), 273 (100), 211 (26), 207 (19), 164 (92), 151 (52), 148 (38), 137 (78), 136 (64), 107 (46). *Anal.* Calcd for $C_{20}H_{24}O_6$: C, 66.65; H, 6.71. Found: C, 66.82; H, 6.69.

9: mp 74—76 °C. UV λ_{max}^{MeOH} nm (log ε): 209 (3.97), 250 (3.73), 305 (4.10). IR ν_{max}^{KBr} cm^{-1}: 3050, 1690, 1650, 1610, 1265, 1150. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, d, J=6 Hz), 2.40 (1H, dd, J=15.0, 13.5 Hz), 2.91 (1H, dd, J=15.0, 6.0 Hz), 3.71 (3H, s), 5.06 (2H, m), 5.93 (1H, br s), 6.19 (1H, s). *Anal.* Calcd for $C_{10}H_{12}O_4$: C, 61.21; H, 6.17. Found: C, 61.47; H, 6.25.

10a: mp 73—75 °C. IR ν_{max}^{KBr} cm^{-1}: 3470, 3050, 1660, 1570, 1500, 1355, 1300, 1250, 1225, 1100, 1000, 840, 790. *Anal.* Calcd for $C_{18}H_{18}O_5$: C, 68.78; H, 5.77. Found: C, 68.89; H, 5.82.

11a: mp 134—136 °C. IR ν_{max}^{KBr} cm^{-1}: 3350, 1720, 1680, 1600, 1500, 1250, 1120, 1055, 1020, 920, 875, 860, 813, 782, 754, 725. *Anal.* Calcd for $C_{16}H_{14}O_6$: C, 63.57; H, 4.67. Found: C, 63.78; H, 4.58.

12a: Oil. IR ν_{max}^{neat} cm^{-1}: 3040, 1750, 1630, 1250, 1190, 1140, 1110, 1020, 890, 850, 820, 775. ^{1}H-NMR ($CDCl_3$) δ: 1.14 (3H, d, J=6.0 Hz), 1.85 (3H, dd, J=6.0, 1.0 Hz), 1.97 and 2.25 (each 3H, s), 3.85 (6H, s), 4.55 (1H, m, ArOC<u>H</u>CH_3), 5.65 (1H, m, CH_3C<u>H</u>=CH–), 5.91 (1H, d, J=6.5 Hz, ArC<u>H</u>OAc), 6.35 (1H, br d, J=15.5 Hz, ArC<u>H</u>=CHCH$_3$), 6.70—7.05 (6H, m). One of the epimers: ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, d, J=6.1 Hz), 1.80 (3H, d, J=6.0 Hz), 1.97 and 2.25 (each 3H, s), 3.85 (6H, s), 4.55 (1H, m), 5.65 (1H, m), 5.91 (1H, d, J=4.7 Hz), 6.35 (1H, br d, J=15.5 Hz), 6.70—7.05 (6H, m). *Anal.* Calcd for $C_{24}H_{28}O_7$: C, 67.27; H, 6.59. Found: C, 67.73; H, 6.50.

Conversion of 3b to 3c by Acid **3b** (10 mg) was dissolved in 1 N HCl MeOH solution (1 ml) and kept at room temperature overnight. The reaction mixture was treated by a usual method to give **3c** (6 mg). Oil. IR ν_{max}^{neat} cm^{-1}: 3450, 1600, 1500, 1240, 1120, 1030, 820. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, t, J=7.1 Hz), 1.36 (3H, d, J=6.0 Hz), 1.57 (2H, m), 2.44 (2H, t, J=7.1 Hz), 3.32, 3.78 and 3.87 (each 3H, s), 4.30 (2H, m, ArC<u>H</u>-C<u>H</u>Ar), 5.60 (1H, br s, –OH), 6.64—6.91 (6H, m). One of the epimers: ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, t, J=7.1 Hz), 1.06 (3H, d, J=6.0 Hz), 1.60 (2H, m), 2.50 (2H, t, J=7.1 Hz), 3.28, 3.82, 3.89 (each 3H, s), 4.34 (2H, m), 6.50 (1H, br s, –OH), 6.64—6.91 (6H, m).

Saponification of 3a by Base **3a** (10 mg) was dissolved in 1 N NaOH MeOH solution (1 ml) and kept at room temperature for 5 h under a nitrogen atmosphere. The reaction mixture was treated by a usual method to give **3c** (6 mg).

Catalytic Hydrogenolysis of 5a, 5d, 7a or 7b Compound **5a** (15 mg), **5d** (18 mg), **7a** (16 mg) or **7b** (18 mg) was dissolved in 5 ml of MeOH, then 10 mg of 5% Pd–C suspended in 5 ml of MeOH was added and the mixture was saturated with H_2. After 1 d, the catalyst was removed by filtration and washed several times with MeOH. After purification, the combined filtrate yielded **13** (11 mg). mp 68—70 °C. IR ν_{max}^{KBr} cm^{-1}: 3630, 1750, 1600, 1520, 1270, 1230, 1115, 1035, 1025, 920, 835, 745. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, d, J=6.1 Hz), 2.28 (3H, s), 3.04 (2H, d, J=6.3 Hz), 3.62 (3H, s), 4.30 (1H, m), 5.16 (1H, br s, –OH), 6.31—6.94 (3H, ABX system).

Dehydration of 13 with Acid **13** (10 mg) and p-toluenesulfonic acid (10 mg) were heated at 50 °C for 6 h in 5 ml of MeOH. Purification yielded a product (5 mg) identical with isoeugenol (**1**).

Acetylation of 5b with Ac_2O and Pyridine at 60 °C **5b** (10 mg) was dissolved in a mixture of 1 ml of Ac_2O and 1 ml of pyridine, and the reaction mixture was heated at 60 °C for 6 h. The usual work-up afforded **5d** (11 mg). Oil. IR ν_{max}^{neat} cm^{-1}: 1760, 1730, 1600, 1510, 1250, 1210, 1120, 1090, 1050, 925. ^{1}H-NMR ($CDCl_3$) δ: 1.16 (3H, d, J=7.0 Hz), 1.23 (3H, t, J=7.0 Hz), 1.97, 2.30, and 3.83 (each 3H, s), 3.43 (2H, m, AB system, –OC<u>H</u>$_2$$CH_3$), 4.31 (1H, d, J=5.1 Hz), 5.00 (1H, m), 6.89—7.03 (3H, m, phenyl protons).

Acetylation of 10a with Ac_2O and Pyridine A solution of **10a** (46 mg) in Ac_2O (1 ml) and pyridine (1 ml) was left overnight at room temperature. The reaction mixture was treated by the usual method to give an oil (**10b**) (40 mg). IR ν_{max}^{neat} cm^{-1}: 3050, 1755, 1680, 1580, 1500, 1250, 1150, 1020, 915, 850, 715. ^{1}H-NMR ($CDCl_3$) δ: 1.48 (3H, d, J=7.0 Hz), 2.28, 3.82 and 3.92 (each 3H, s), 3.52 (1H, m), 5.28 (1H, d, J=10.1 Hz), 6.89—7.38 (5H, m, phenyl protons), 9.83 (1H, s).

Sodium Borohydride Reduction of 10a An excess of sodium borohydride (50 mg) was added in small portions to a solution of **10a** (60 mg) in 1 ml of MeOH, and after 4 h the solution was poured into water (30 ml). The product (**15**) had mp 84—85 °C (42 mg). IR ν_{max}^{KBr} cm^{-1}: 3390, 1598, 1500, 1260, 1200, 1120, 1010, 930, 810, 730. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (3H, d, J=7.0 Hz), 3.51 (1H, m), 3.92 and 3.98 (each 3H, s), 4.68 (2H, s), 5.18 (1H, d, J=10.0 Hz), 5.95 (1H, br s, phenolic –OH), 6.78—6.92 (3H, m, phenyl protons), 7.01 (2H, br s, phenyl protons).

Photooxidation of 2 in Acetonitrile A solution of **2** (500 mg) and methylene blue (10 mg) in acetonitrile (20 ml) was irradiated with a fluorescent lamp. The reaction was continued for 3 d at 10—15 °C and

gave **10a** (30 mg).

Conversion of 11a to 6 and 4a **11a** (45 mg) and *p*-toluenesulfonic acid (5 mg) were dissolved in 20 ml of MeOH and heated under reflux for 8 h. The product was purified by silica gel chromatography to give two products **4a** (20 mg) and **6** (18 mg).

Acetylation of 8a and 11a Acetylation of **8a** and **11a** by using the above-mentioned method afforded **8b**: mp 180—182 °C. IR ν_{max}^{KBr} cm^{-1}: 1770, 1600, 1500, 1270, 1180, 1150, 1125, 1060, 1035, 935, 915, 835, 768. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (6H, d, J=6.1 Hz), 2.25 and 3.64 (each 6H, s), 2.68 and 4.59 (each 2H, m), 6.34 (2H, d, J=1.7 Hz), 6.60 (2H, dd, J=8.1, 1.7 Hz), 6.84 (2H, d, J=8.1) and **11b**: mp 158—160 °C. IR ν_{max}^{KBr} cm^{-1}: 1750, 1735, 1670, 1600, 1500, 1235, 1160, 1065, 1025, 900, 845, 770, 755. ^{1}H-NMR ($CDCl_3$) δ: 2.34, 3.89 and 3.92 (each 3H, s), 7.11—7.89 (6H, m), 9.90 (1H, s), respectively.

Conversion of 12a to 3c **12a** (10 mg) and PtO_2 (5 mg) were added to 3 ml of MeOH, then hydrogen was bubbled through the solution under stirring. After 4 h, the reaction mixture was filtered and 5 mg of *p*-toluenesulfonic acid was added to the filtrate. The mixture was kept at room temperature overnight. After purification by silica gel chromatography, it afforded **3c** (5 mg).

Photooxidation of 6 in MeOH and EtOH Vanillin (**6**) (8 g) was oxidized with singlet oxygen under the conditions mentioned above. After 6 d, it gave **4a** (30 mg), **4b** (35 mg), and **11a** (40 mg) from MeOH, EtOH, and acetone solutions, respectively.

Autooxidation of 6 in MeOH and EtOH A solution of vanillin (5 g) and *p*-toluenesulfonic acid (0.1 g) in 50 ml of MeOH or EtOH was oxidized with air in the dark. After 7 d, it gave **4a** (5 mg) and **4b** (7 mg) from the MeOH and EtOH solutions, respectively.

Acknowledgement This research was supported by the National Science Council of the R.O.C.

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Stereoselective Reactions. XX.[1] Synthetic Studies on Optically Active β-Lactams. III.[2] Stereocontrolled Synthesis of Chiral Intermediate to (+)-Thienamycin from D-Glucose[3]

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A chiral key intermediate (19a) for the synthesis of (+)-thienamycin was synthesized starting from D-glucose. The enol ether 13, obtained from the ketone 11 by Horner–Wittig reaction, was transformed to the corresponding methyl ester 16 by pyridinium chlorochromate oxidation or by employing the Wacker process. The ester 16 was further converted to the β-lactam 19a, which is a useful chiral precursor to (+)-thienamycin.

Keywords β-lactam; thienamycin; chiral synthesis; D-glucose; Horner–Wittig reaction; pyridinium chlorochromate; Wacker process

Thienamycin (**1**),[5] a β-lactam antibiotic having a 1-carbapen-2-em ring system and three contiguous chiral centers at C-5, 6 and 8, is reported to exhibit a desirable antibacterial activity as well as stability to β-lactamase. A number of syntheses of (+)-**1** and the key intermediates for (+)-**1** have been reported from a variety of starting materials such as (*S*)-aspartic acid,[6a] penicillin,[6b] (*S*)-threonine,[6c] D-glucose derivatives,[3,6d-f] (*S*)-3-hydroxybutanoate derivatives,[6g] and by asymmetric induction.[6h-j]

As shown in Chart 1, retrosynthetic analysis of thienamycin has led to the recognition of a hidden D-glucose skeleton within the thienamycin molecule. Thus, successive bond disconnection of thienamycin at the double bond (C-2–C-3) and β-lactam function (N-4–C-7) generates a β-amino acid **2**, which can be related to the δ-lactone **3**. Since racemic **3** has already been converted into (±)-thienamycin,[7] optically pure **3** was selected as the target for the chiral synthesis of **1**. The δ-lactone **3** could be obtained by functionalization of the 3- and 4-hydroxy groups of 2,6-dideoxy-α-D-*arabino*-pyranose (**4**), which in turn can be prepared from D-glucose. The details are presented in this paper.

The synthetic scheme for the preparation of (+)-thienamycin is illustrated in Charts 2 and 3. The starting material, methyl 3-azido-4-*O*-benzoyl-6-bromo-2,3,6-trideoxy-α-D-*arabino*-hexopyranoside (**6**), which already contains a 2-deoxy function and the potential amino group at C-3, was obtained from D-glucose.[8,9] Hydrogenation of the azido group, reductive dehalogenation and debenzoylation were effected in a single step by treatment of **6** with Raney nickel under hydrogen in the presence of triethylamine (TEA) in methanol to furnish the corresponding amino alcohol, which, without purification, was converted into various *N*-protected derivatives (**7a**, **c**, **d**) by treatment with acetic anhydride, trifluoroacetic anhydride, and benzyloxycarbonyl (Z) chloride, respectively, in 48—57% yields. The next stage of the synthesis was to introduce a carboxyl group or its equivalent at C-4 of **7a**—**d**. First, the introduction of the cyano group as a carboxyl equivalent was examined using potassium cyanide. Since the attempt to convert the equatorial 4-*O*-methanesulfonate **7b** into the corresponding nitrile **7e** with potassium cyanide in *N*,*N*-dimethylformamide (DMF) at 100 °C by a double-inversion process *via* the oxazolinium intermediate, involving participation of the neighboring acetamide group, resulted in the formation of the corresponding 1,3-oxazoline as the sole product, the axial 4-hydroxy derivatives (**8a** and **8d**) were prepared from **7a** and **7c**, and converted to 4-*O*-

1 2 3 4 5

Chart 1

D-glucose → 6 → 7 → 8; 9; 10

7a : R^1=OH, R^2=Ac
7b : R^1=OMs, R^2=Ac
7c : R^1=OH, R^2=TFA
7d : R^1=OH, R^2=Z
7e : R^1=CN, R^2=Ac

8a : R^1=H, R^2=Ac
8b : R^1=Ms, R^2=Ac
8c : R^1=BBS, R^2=Ac
8d : R^1=H, R^2=TFA
8e : R^1=BBS, R^2=TFA
8f : R^1=H, R^2=BBS

TFA : CF_3COO-
Z : $C_6H_5CH_2OCO$-
Ms : CH_3SO_2-
BBS : *p*-$BrC_6H_4SO_2$-

Chart 2

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12a : major product
12b : minor product

13a : R^1=H, R^2=CH_3O-
13b : R^1=CH_3O-, R^2=H

17a : R^1=Z, R^2=CH_3
17b : R^1=R^2=H

Bn : $C_6H_5CH_2$-

19a : R=Bn
19b : R=H

20a : X=2H
20b : X=N_2

PNB : *p*-$NO_2C_6H_4CH_2$-

Chart 3

Fig. 1

methanesulfonyl and 4-*O*-*p*-bromobenzenesulfonyl derivatives (**8b**, **c**, **e**) according to the reported procedure,[9,10] and then the introduction of a cyano group in an *S*N2 fashion was examined. However, the reaction of **8b** and **8c** with potassium cyanide in the presence of 18-crown-6 in acetonitrile at reflux temperature afforded the olefinic compound (**9**) and the *N*-*p*-bromobenzenesulfonyl derivative (**8f**), respectively. Interestingly, the 3-cyano derivative (**10**) was obtained by the reaction of **8e** with potassium cyanide in the presence of 18-crown-6 in dimethyl sulfoxide (DMSO) at 60—80 °C in 66% yield.[11]

Since one of the efficient sequences for the one-carbon homologation of ketone to carboxylic acid or its equivalent is the Horner–Wittig reaction, the preparation of the enol ether **13** from the ketone **11** derived from the alcohol **7d** and its conversion to a carboxyl compound were examined by employing the Horner–Wittig reaction. Oxidation of **7d** with trifluoroacetic anhydride and DMSO in methylene dichloride[12] afforded the corresponding ketone **11** in 95% yield, and **11** was treated with the anion derived from methoxymethyldiphenylphosphine oxide[13] and lithium diisopropylamide (LDA) in tetrahydrofuran (THF) to give the adducts **12** as a mixture of two diastereomers in a ratio of 2.8 : 1 in 92% yield. The isomers were separated by column chromatography and treated with potassium hydride in DMF to provide the enol ethers **13a** and **13b** in 54—75% yields. The major diastereomer **12a** was converted to **13a**. Regiochemistry of **13a** and **13b** was tentatively determined by means of proton nuclear Overhauser effect (NOE) experiments. Upon irradiation of the vinyl proton of **13a**, significant enhancement (about 15%) of the C-6 methyl proton signal was observed, while no such enhancement was observed for **13b**. The conversion of the enol ether **13a** to the corresponding aldehyde **15** by acid hydrolysis resulted in the formation of the undesired ene-dialdehyde **14** by β-elimination. However, oxidation of the enol ethers **13a** and **13b** with pyridinium chlorochromate (PCC) in methylene dichloride[14] gave the corresponding methyl ester **16** (mp 128 °C, $[\alpha]_D^{20}$ +79.6° ($CHCl_3$)) in 30 and 15% yields, respectively. Stable equatorial orientation of the methoxycarbonyl group at C-4 is supported by the nuclear magnetic resonance (NMR) coupling constant (10 Hz) between H-4 and H-5, and no epimer at C-4 could be detected. A possible reaction mechanism is shown in Fig. 1. Initial electrophilic attack on the olefin by PCC from the less-hindered α-side gave an unstable intermediate **23**, in which heterolytic cleavage of the Cr–O bond, accompanied by a 1,2-hydride shift on the β-side gave the

TABLE I. Conversion of the Enol Ether (**13a**) to the Methyl Ester (**16**) with Various Reagents

Oxidant (eq)	Solvent	Temperature	Time (h)	Yield of **16** (%)
PCC (3)	CH_2Cl_2	r.t.	24	30
PDC (2)	DMF	r.t.	48	7
Collins reagent (7.5)	CH_2Cl_2	r.t.	48	No reaction
Jones reagent (2)	Acetone	0 °C	2	Many products
$PdCl_2$ (0.1) $CuCl_2$ (1)	$DMF-H_2O$ (10 : 1)	r.t.	24	No reaction
$PdCl_2$ (0.5) CuCl (1)	$DMF-H_2O$ (10 : 1)	r.t.	20	25
$PdCl_2$ (0.5) CuCl (5)	$DMF-H_2O$ (10 : 1)	70 °C	19	53

r.t. = room temperature.

Fig. 2

methoxycarbonyl compound with equatorial orientation. The use of other chrominium oxidants such as pyridinium dichromate (PDC), Collins reagent, and Jones reagent did not improve the conversion yield of **13a** to **16**. However, by employing the Wacker process[15] using 0.5 eq of palladium chloride and 5 eq of cuprous chloride in $DMF-H_2O$ (10 : 1) at 70 °C for 19 h, **16** was obtained in 53% yield from **13a**. In this reaction too, no epimer at C-4 was detected. The results on the conversion of **13a** to **16** are summarized in Table I. Figure 2 shows a possible reaction mechanism *via* the Wacker process. The palladium reagent approaches the olefin from the less-hindered α-side to form the π-complex **24**. Addition of water to the double bond forms the unstable σ-alkyl derivative (**25**), which was converted to the methyl ester **16** by a 1,2-hydride shift and cleavage of the Pd–C bond.

The ester **16** was treated with aqueous HCl in THF followed by Jones oxidation to provide the δ-lactone **17a** in 65% yield. The δ-lactone **17a** was further converted to the chiral thienamycin derivative (**22**) *via* the β-lactam **19** by employing Merck's procedure.[7] Removal of the Z group by catalytic hydrogenation followed by acid hydrolysis of the methyl ester gave the lactone acid **17b**, which was heated in benzyl alcohol to afford an equilibrium mixture of the acyclic β-amino acid hydrochloride **18** and starting lactone acid **17b**. This was used, without isolation, for β-lactam ring formation with *N,N'*-dicyclohexylcarbodiimide to give the β-lactam **19a** having the requisite chirality in 64% yield from **17a** after purification by column chromatography. After removal of the benzyl group of **19a**, the resulting acid **19b** was treated with *N,N'*-carbonyldiimidazole, followed by addition of the magnesium salt of *p*-nitrobenzyl hydrogen malonate[16] to afford the β-ketoester **20a** in 74% yield from **19a**. The construction of the carbapenam ring system by carbene insertion reaction of the diazo keto ester **20b** with a catalytic amount of rhodium acetate in benzene gave the bicyclic ketone **21**. Then, the ketone **21** was treated with diphenyl phosphorochloridate and *N,N*-diisopropylethylamine in acetonitrile to provide the corresponding enol phosphate, which was reacted with *N-p*-nitrobenzyloxycarbonylaminoethanthiol to afford the bis-protected thienamycin **22** (mp 173.5—175 °C, $[\alpha]_D^{20}$ +59.5° (DMF)), which was precipitated directly from the reaction mixture in 48% yield from **20a**. Its spectral data are identical with those reported for racemic **22**.[7] The bis-protected thienamycin **22** was previously transformed into (+)-thienamycin by catalytic hydrogenation.[6a,7,18]

The presently described synthesis should be totally stereoselective and provide optically pure thienamycin. Further synthetic studies on optically active thienamycin derivatives are in progress in our laboratory.

Experimental

All melting temperatures are uncorrected. Infrared (IR) spectral measurements were performed with a JASCO DS-402G or a JASCO IRA-1 grating IR spectrometer. ^{1}H-NMR spectra were measured with JEOL JNM-FX 100 (100 MHz), JEOL JNM-PS (100 MHz) and Hitachi R-24 (60 MHz) spectrometers. ^{13}C-NMR spectra were measured with a JNM-FX 100 (100 MHz) spectrometer. Data are recorded in parts per million (ppm) downfield from internal tetramethylsilane. The following abbreviations are used: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Optical rotations were determined with a JASCO DIP-181 polarimeter. Mass spectra (MS) were recorded with a JEOL JMS-01 5G-Z mass spectrometer. The organic solvents were dried over $MgSO_4$ before vacuum evaporation.

Methyl 3-Azido-4-*O*-benzoyl-6-bromo-2,3,6-trideoxy-α-D-*arabino*-hexopyranoside (6) This sample was obtained from methyl 3-azido-4,6-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside by the reported procedure,[8] mp 65—65.5 °C (2-propanol), $[\alpha]_D^{20}$ +41.9° (c = 1.0, $CHCl_3$), (lit.[8] syrup, $[\alpha]_D^{25}$ +52.7° (c = 0.53, $CHCl_3$)).

Reaction of Methyl 3-Acetamido-4-*O*-methanesulfonyl-2,3,6-trideoxy-α-D-*lyxo*-hexopyranoside (8b) with Potassium Cyanide A mixture of methyl 3-acetamido-2,3,6-trideoxy-α-D-*lyxo*-hexopyranoside (**8a**, 60 mg, 0.3 mmol, mp 176.5—178 °C; $[\alpha]_D^{20}$ +219° (c = 1.88, MeOH), lit.[9] mp 176—178 °C; $[\alpha]_D^{20}$ +220° (c = 1.45, MeOH)) and methanesulfonyl chloride (44 mg, 0.38 mmol) in pyridine (1 ml) was stirred at −20 °C overnight. After removal of the solvent *in vacuo*, the residue was purified by column chromatography (silica gel, $CHCl_3$: acetone = 20 : 1) to afford **8b** (42 mg, yield 51%) as crystals, mp 173—174.5 °C (2-propanol). ^{1}H-NMR ($CDCl_3$): 1.22 (3H, d, J = 7 Hz, C_6-CH_3), 1.6—2.34 (2H, m, C_2-H), 1.96 (3H, s, $COCH_3$), 3.12 (3H, s, SO_2CH_3), 3.31 (3H, s, OCH_3), 3.76—4.92 (4H, m, C_1-, C_3-, C_4-, and C_5-H), 6.27 (1H, s, NH). MS m/z: 281 (M^+).

A mixture of **8b** (20 mg, 0.07 mmol), potassium cyanide (23 mg, 0.35 mmol), and 18-crown-6 (9.4 mg, 0.035 mmol) in acetonitrile (1 ml) was heated under reflux for 10.5 h. After removal of the solvent *in vacuo*, an oily residue was purified by column chromatography (silica gel, $CHCl_3$: acetone = 30 : 1) to afford **9** (5 mg, yield 39%) and starting **8b** (11 mg, 55% recovery). **9**: oil, IR ν_{max}^{film} cm^{-1}: 1620. ^{1}H-NMR ($CDCl_3$): 1.76 (3H, s, C_6-CH_3), 1.95 (3H, s, $COCH_3$), 1.6—2.15 (2H, m, C_2-H), 3.69 (3H, s, OCH_3), 4.40—5.70 (3H, m, C_1-, C_3-, and C_4-H). MS *m/z*: 185 (M^+).

Reaction of Methyl 3-Acetamido-4-*O*-*p*-bromobenzenesulfonyl-2,3,6-trideoxy-α-D-*lyxo*-hexopyranoside (8c) with Potassium Cyanide A mixture of **8c** (41 mg, 0.096 mmol, mp 182—184 °C; $[\alpha]_D^{20}$ +217° (c = 1, MeOH)), potassium cyanide (39 mg, 0.6 mmol), and 18-crown-6 (16 mg, 0.06 mmol) in acetonitrile (1 ml) was heated under reflux for 4.5 h. After removal of the solvent *in vacuo*, the resulting residue was dissolved in $CHCl_3$. The insoluble materials were filtered off and the filtrate was concentrated *in vacuo* to give a residue, which was purified by column chromatography (silica gel, benzene : acetone = 5 : 1) to afford **8f** (35 mg, yield 95%) as crystals, mp 176 °C (2-propanol). IR ν_{max}^{KBr} cm^{-1}: 3320, 3300, 1575. ^{1}H-NMR ($CDCl_3$): 1.21 (3H, d, J = 7 Hz, C_6-CH_3), 1.4—1.9 (2H, m, C_2-H_{ab}), 2.55 (1H, br s, OH), 3.29 (3H, s, OCH_3), 3.1—4.05 (3H, m, C_3-, C_4-, C_5-H), 4.5—4.74 (1H, m, C_1-H), 6.08 (1H, br s, NH), 7.4—7.9 (4H, m, aromatic protons). *Anal.* Calcd for $C_{13}H_{18}BrNO_5S$: C, 41.07; H, 4.77; N, 3.68. Found: C, 41.00; H, 4.71; N, 3.81.

Reaction of Methyl 4-*O*-*p*-Bromobenzenesulfonyl-3-trifluoroacetamido-2,3,6-trideoxy-α-D-*lyxo*-hexopyranoside (8e) with Potassium Cyanide A mixture of **8e** (150 mg, 0.32 mmol, prepared from **7c**[10b] in the same manner as described above for the preparation of **8c**, mp 177—179 °C, $[\alpha]_D^{20}$ +115° (c = 1.1, $CHCl_3$)), potassium cyanide (103 mg, 1.58 mmol), and 18-crown-6 (40 mg, 0.15 mmol) in DMSO (1 ml) was heated at 60 °C for 19 h, then at 80 °C for 13 h. After removal of the solvent *in vacuo*, the residue was dissolved in acetone and $CHCl_3$ (1 : 1). The insoluble materials were filtered off and the filtrate was concentrated *in vacuo* to give an oily residue, which was purified by column chromatography (silica gel, hexane : ether = 2 : 1) to afford **10** (55 mg, yield 66%) as an oil. IR: ν_{max}^{film} cm^{-1}: 3350, 2250 (CN), 1738. ^{1}H-NMR ($CDCl_3$): 1.22 (3H, d, J = 6 Hz, C_6-CH_3), 1.71 (1H, dd, J = 12, 15 Hz, C_4-H_a), 2.25 (2H, m, C_2-H_{ab}), 2.59 (1H, dd, J = 2, 15 Hz, C_4-H_e), 3.34 (3H, s, OCH_3), 3.78 (1H, ddq, J = 2, 6, 12 Hz, C_5-H), 4.83 (1H, m, C_1-H), 8.02 (1H, br s, NH). ^{13}C-NMR ($CDCl_3$): 20.4 (q, C_6), 36.7 (t), 37.7 (t), 47.6 (s, C_3), 55.4 (q, OCH_3), 59.7 (d, C_5), 96.9 (d, C_1), 115.4 (q, CF_3), 117.5 (s, CN), 115.9 (q, CO). High-resolution MS *m/z* ($(M+1)^+$): Calcd for $C_{10}H_{13}F_3N_2O_3$: 265.0799. Found: 265.0799.

Methyl 3-(Benzyloxycarbonyl)amino-2,3,6-trideoxy-α-D-*arabino*-hexopyranoside (7d) A mixture of **6** (10 g, 27 mmol), Raney nickel (W-4, 20 g), and TEA (2.76 g, 27.3 mmol) in MeOH (80 ml) was shaken under hydrogen at 3.6 kg/cm^2 for 8 h. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo* to give a residue, which was treated with benzyloxycarbonyl chloride (4.2 ml, 29.4 mmol) and TEA (4.3 ml, 30.9 mmol) in CH_2Cl_2 (100 ml) at room temperature for 4 h. After successive washing with saturated aqueous $NaHCO_3$, H_2O, and saturated aqueous NaCl, drying followed by evaporation gave a residue, which was purified by column chromatography (silica gel, benzene : AcOEt = 3 : 1) to afford **7d** (4.3 g, yield 55%) as crystals, mp 132—135 °C (benzene), $[\alpha]_D^{22}$ +117.4° (c = 1.4, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3410, 3305, 1680. ^{1}H-NMR ($CDCl_3$): 1.27 (3H, d, J = 6 Hz, C_6-CH_3), 1.63 (1H, ddd, J = 3, 13, 13 Hz, C_2-H_a), 2.01 (1H, dd, J = 4, 13 Hz, C_2-H_e), 3.30 (3H, s, OCH_3), 2.70—4.22 (4H, m, C_3-, C_4-, and C_5-H, OH), 4.64 (1H, d, J = 3 Hz, C_1-H), 5.04 (2H, s, OCH_2Ph), 5.32 (1H, d, J = 7 Hz, NH), 7.28 (5H, s, aromatic protons). *Anal.* Calcd for $C_{15}H_{21}NO_5$: C, 61.00; H, 7.17; N, 4.74. Found: C, 61.14; H, 7.30; N, 4.63.

Methyl 3-(Benzyloxycarbonyl)amino-2,3,6-trideoxy-α-*arabino*-hexopyranosid-4-ulose (11) A solution of trifluoroacetic anhydride (5.7 ml, 40.6 mmol) in CH_2Cl_2 (13 ml) was added at −78 °C to a solution of DMSO (3.84 ml, 49 mmol) in CH_2Cl_2 (95 ml) over a period of 10 min. The mixture was stirred at −78 °C for 15 min, then a solution of **7d** (8.0 g, 27.1 mmol) in CH_2Cl_2 (95 ml) was added below −65 °C over a period of 20 min. The mixture was stirred below −65 °C for 1.5 h, followed by addition of TEA (11.3 ml, 81.2 mmol). After warming to room temperature, the mixture was washed with H_2O (100 ml). The aqueous layer was back-washed with CH_2Cl_2. The combined organic solutions were washed with saturated aqueous NaCl. Drying followed by evaporation gave a residue, which was purified by column chromatography (silica gel, benzen : AcOEt = 20 : 1) to give a solid. Recrystallization from isopropyl ether–hexane provided **11** (7.5 g, yield 95%) as prisms, mp 82—83 °C, $[\alpha]_D^{20}$ +91.2° (c = 1, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3290, 1730, 1650. ^{1}H-NMR ($CDCl_3$): 1.20 (3H, d, J = 6 Hz, C_6-CH_3), 1.86 (1H, ddd, J = 4, 14, 15 Hz, C_2-H_a), 2.64 (1H, ddd, J = 7, 7, 15 Hz, C_2-H_e), 3.34 (3H, s, OCH_3), 4.28 (1H, q, J = 7 Hz, C_5-H), 4.43—5.23 (2H, m, C_1- and C_3-H), 5.01 (2H, s, OCH_2Ph), 5.83 (1H, d, J = 7 Hz, NH), 7.20 (5H, s, aromatic protons). *Anal.* Calcd for $C_{15}H_{19}NO_2$: C, 61.42; H, 6.53; N, 4.78. Found: C, 61.35; H, 6.52; N, 4.60.

Methyl 3-(Benzyloxycarbonyl)amino-4-(diphenylphosphinoylmethoxy)-methyl-2,3,6-trideoxy-α-D-*arabino*-hexopyranoside (12) A solution of methoxymethyldiphenylphosphine oxide (12 g, 48.8 mmol) in anhydrous THF (140 ml) was added to LDA in anhydrous THF (140 ml) prepared from diisopropylamine (6.9 ml, 49.3 mmol) and butyllithium (32.2 ml of a 1.52 M solution in hexane) below −65 °C over a period of 10 min, and the mixture was stirred below −65 °C for 20 min. Then, a solution of **11** (7.0 g, 23.9 mmol) in THF (60 ml) was added below −65 °C over a period of 20 min. After being stirred below −65 °C for 1.5 h, the mixture was allowed to warm to −10 °C, then the reaction was quenched with 10% aqueous citric acid (100 ml). The organic layer was separated, and the aqueous layer was extracted with AcOEt (100 ml × 3). The combined organic layers were washed with saturated aqueous NaCl. Drying followed by evaporation gave a residue, which was purified by column chromatography (silica gel, AcOEt) to provide **12** (11.9 g, yield 92%) as a mixture of two diastereomers. They were separated by medium-pressure liquid chromatography (silica gel, AcOEt). Less polar isomer (**12a**, oil, 7.84 g): $[\alpha]_D^{20}$ +60.6° (c = 1.04, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 3300, 1705, 1125, 1110. ^{1}H-NMR ($CDCl_3$): 1.15 (3H, d, J = 7 Hz, C_6-CH_3), 1.67—2.1 (2H, m, C_2-H), 3.23 (3H, s, POCHOC$\underline{H}_3$), 3.26 (3H, s, OCH_3), 3.78—4.72 (4H, m, C_1-, C_3-, and C_5-H, POCH), 5.06 (2H, s, OCH_2), 5.22 (1H, br s, OH), 5.50 (1H, d, J = 10 Hz, NH), 7.0—8.20 (15H, m, aromatic protons). MS *m/z*: 539 (M^+). Polar isomer (**12b**, oil, 2.8 g): $[\alpha]_D^{20}$ +72.2° (c = 1, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 3300, 1708, 1127, 1111. ^{1}H-NMR ($CDCl_3$): 1.19 (3H, d, C_6-CH_3), 1.70—2.10 (2H, m, C_2-H), 3.15 (3H, s, POCHOC$\underline{H}_3$), 3.20 (3H, s, OCH_3), 3.70—5.20 (6H, m, C_1-, C_3-, and C_5-H, POCH, OCH_2), 5.75 (1H, d, J = 8 Hz, NH), 6.90—8.20 (15H, m, aromatic protons). MS *m/z*: 539 (M^+).

Methyl 3-(Benzyloxycarbonyl)amino-4-(*E*)-methoxymethylene-2,3,4,6-tetradeoxy-α-D-*arabino*-hexopyranoside (13a) A solution of **12a** (747 mg, 1.38 mmol) in DMF (5 ml) was added to a solution of potassium hydride (480 mg, 4.2 mmol, 35% oil suspension, washed with hexane) in DMF (10 ml) at −10 °C over a period of 20 min. The mixture was stirred at 0 °C for 1 h, then 10% aqueous citric acid (20 ml) was added, and the whole was extracted with AcOEt (50 ml × 4). The combined organic extracts were washed with saturated aqueous NaCl. Drying followed by evaporation gave a residue, which was purified by column chromatography (silica gel, benzene : AcOEt = 30 : 1) to afford a solid (390 mg). Recrystallization from hexane gave **13a** (333 mg, yield 75%) as colorless needles, mp 66—67 °C, $[\alpha]_D^{20}$ +86.9° (c = 1.0, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3325, 1700. ^{1}H-NMR ($CDCl_3$): 1.39 (3H, d, J = 6 Hz, C_6-CH_3), 1.7—1.85 (1H, m, C_2-H_a), 2.12—2.45 (1H, ddd, J = 4.9, 14 Hz, C_2-H_e), 3.36 (3H, s, C_1-OCH_3), 3.57 (3H, s, OCH_3), 4.12—4.38 (1H, m, C_3-H), 4.66 (1H, dq, J = 2, 7 Hz, C_5-H), 4.87 (1H, dd, J = 4, 8 Hz, C_1-H), 5.09 (2H, s, OCH_2), 5.33 (1H, d, J = 8 Hz, NH), 6.01 (1H, m, C$\underline{H}$OCH$_3$), 7.35 (5H, s, aromatic protons). ^{13}C-NMR ($CDCl_3$): 20.7 (q, C_6), 37.2 (t, C_2), 46.9 (d, C_3), 55.2 (q, OCH_3), 60.0 (d, C_5), 63.8 (q, CHO$\underline{C}$H$_3$), 66.6 (t, OCH_2), 97.1 (d, C_1), 116.4 (s, C_4), 128.0, 128.4, and 136.6 (aromatic carbons), 143.0 (d, =$\underline{C}$HOCH$_3$), 155.4 (s, CO). *Anal.* Calcd for $C_{17}H_{23}NO_5$: C, 63.54; H, 7.21; N, 4.36. Found: C, 63.49; H, 7.26; N, 4.34.

Methyl 3-(Benzyloxycarbonyl)amino-4-(*Z*)-methoxymethylene-2,3,4,6-tetradeoxy-α-D-*arabino*-hexopyranoside (13b) This sample was obtained from **12b** as an oil in 54% yield in the same manner as described for the preparation of **13a**, $[\alpha]_D^{20}$ +97.4° (c = 2.9, $CHCl_3$). IR ν_{max}^{film} cm^{-1}: 3340, 1715. ^{1}H-NMR ($CDCl_3$): 1.25 (3H, d, J = 7 Hz, C_6-CH_3), 1.5—2.3 (2H, m, C_2-H), 3.35 (3H, s, C_1-OCH_3), 3.54 (3H, s, =CHOC$\underline{H}_3$), 4.15—4.50 (1H, m, C_3-H), 4.58—4.95 (2H, m, C_1- and C_5-H), 5.09 (2H, s, OCH_2), 5.90 (1H, s, =CHO), 7.32 (5H, s, aromatic protons). ^{13}C-NMR ($CDCl_3$): 17.9 (q, C_6), 36.1 (t, C_2), 45.5 (d, C_3), 54.6 (q, OCH_3), 60.1 (d, C_5), 63.8 (q, =CHO$\underline{C}$H$_3$), 66.2 (t, OCH_2), 98.2 (d, C_1), 117.2 (s, C_4), 127.9, 128.3, and 136.8 (aromatic carbons), 142.7 (d, =$\underline{C}$HOMe), 155.6 (s, CO). MS *m/z*: 321 (M^+).

Acid Hydrolysis of 13a A mixture of **13a** (23 mg, 0.072 mmol), 5 N aqueous H_2SO_4 (0.075 ml), and THF (0.3 ml) was stirred at room temperature for 3.5 h, then 0.075 ml of 5 N aqueous H_2SO_4 was added, and the mixture was stirred at room temperature for 4.5 h. After dilution with AcOEt, the mixture was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl. Drying followed by evaporation gave an

oily residue, which was purified by column chromatography (silica gel, benzene : AcOEt = 20 : 1) to afford **14** (7.5 mg, yield 40%) as an oil. ^{1}H-NMR ($CDCl_3$): 2.20 (3H, d, J=6 Hz, CH_3), 2.93 (2H, dd, J=2 and 7 Hz, $C\underline{H}_2CHO$), 5.0—5.25 (1H, m, $C\underline{H}NHZ$), 5.06 (2H, s, $C\underline{H}_2C_6H_5$), 5.82 (1H, d, J=8 Hz, NH), 6.72 (1H, q, J=6 Hz, $CH_3C\underline{H}$), 7.33 (5H, s, C_6H_5), 9.29 (1H, d, J=2 Hz, CHO), 9.67 (1H, t, J=1 Hz, CHO). MS m/z: 275 (M^+).

Methyl 3-(Benzyloxycarbonyl)amino-4-methoxycarbonyl-2,3,4,6-tetradeoxy-α-D-*arabino*-hexopyranoside (16) A) PCC Oxidation: A mixture of **13a** (250 mg, 0.78 mmol) and pyridinium chlorochromate (503 mg, 2.34 mmol) in CH_2Cl_2 (2.5 ml) was stirred at room temperature for 24 h. After addition of ether (10 ml), the mixture was stirred at room temperature for 15 min, then the insoluble materials were filtered off and washed with ether. The combined organic layers were concentrated *in vacuo* to give a residue, which was purified by column chromatography (silica gel, benzene : AcOEt = 15 : 1) to afford **16** (78.6 mg, yield 30%) as crystals, mp 128 °C (isopropyl ether), $[\alpha]_D^{20}$ +79.6° (c=1.1, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3320, 1718, 1680. ^{1}H-NMR ($CDCl_3$): 1.17 (3H, d, J=6 Hz, C_6-CH_3), 1.65 (1H, ddd, J=3, 13, 13 Hz, C_2-H_a), 1.93—2.40 (2H, m, C_2-H_e, C_4-H), 3.32 (3H, s, OCH_3), 3.60 (3H, s, $COOCH_3$), 3.8—4.46 (2H, m, C_3-H and C_5-H), 4.75 (1H, d, J=3 Hz, C_1-H), 4.93 (1H, d, J=10 Hz, NH), 5.02 (2H, s, OCH_2), 7.30 (5H, s, aromatic protons). ^{13}C-NMR ($CDCl_3$): 19.5 (q, C_6), 35.7 (t, C_2), 46.4 (d, C_3), 51.9 (q), 54.6 (q), 55.8 (d), 65.1 (d), 66.6 (t, $\underline{C}H_2Ph$), 97.9 (d, C_1), 128.0, 128.4, and 136.5 (aromatic carbons), 155.3 (s, NHCO), 172.1 (s, $\underline{C}OOCH_3$). *Anal.* Calcd for $C_{17}H_{23}NO_6$: C, 60.52; H, 6.87; N, 4.15. Found: C, 60.61; H, 6.93; N, 4.39.

B) Wacker Process: A mixture of $PdCl_2$ (28 mg, 0.155 mmol) and CuCl (154 mg, 1.56 mmol) in DMF (1 ml) and H_2O (0.1 ml) was stirred at room temperature for 1 h under an oxygen atmosphere, then **13a** (100 mg, 0.31 mmol) was added. The mixture was stirred at 70 °C for 19 h, and the insoluble material was removed by filtration using Celite. After addition of H_2O (3 ml) to the filtrate, the mixture was extracted with AcOEt, and the organic extracts were washed with saturated aqueous $NaHCO_3$. Drying followed by evaporation gave a residue, which was purified by column chromatography (silica gel, benzene : AcOEt = 15 : 1) to afford **16** (55.6 mg, yield 53%) as crystals, whose spectral data were identical with those of the sample obtained by PCC oxidation.

(3*R*,4*S*,5*R*)-3-(Benzyloxycarbonyl)amino-4-methoxycarbonyl-5-hexanolide (17a) A mixture of **16** (370 mg, 1.1 mmol), 0.6 N aqueous HCl (7 ml), and THF (7 ml) was heated under reflux for 3 h. After cooling to room temperature, the mixture was neutralized with aqueous $NaHCO_3$, then extracted with AcOEt (×4). The combined organic extracts were washed with saturated aqueous NaCl. Drying followed by evaporation gave a residue, which was oxidized with Jones reagent (0.41 ml) in acetone (18.5 ml) at 0 °C for 40 min. After addition of 2-propanol (0.24 ml), the mixture was neutralized with $NaHCO_3$, then the organic layer was removed *in vacuo* to give a residue, to which AcOEt (30 ml) and H_2O (5 ml) were added. After removal of the insoluble materials by filtration, the organic layer was separated and washed with saturated aqueous NaCl. Drying followed by evaporation gave a solid, which was recrystallized from benzene–isopropyl ether to give **17a** (228 mg, yield 65%) as colorless needles, mp 118.5—119 °C, $[\alpha]_D^{20}$ +2.2° (c=1.2, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3365, 1720. ^{1}H-NMR ($CDCl_3$): 1.38 (3H, d, J=6 Hz, C_6-CH_3), 2.38—2.79 (2H, m, C_3-H_a, C_5-H), 3.0 (1H, dd, J=7, 15 Hz, C_3-H_e), 3.69 (3H, s, $COOC\underline{H}_3$), 4.13—4.62 (2H, m, C_4- and C_6-H), 5.08 (2H, s, $OC\underline{H}_2Ph$), 5.22 (1H, d, J=8 Hz, NH), 7.33 (5H, s, aromatic protons). ^{13}C-NMR ($CDCl_3$): 19.6 (q, C_7), 35.4 (t, C_3), 47.3 (d, C_4), 52.4 (d, C_5), 52.8 (q, $COO\underline{C}H_3$), 66.8 (t, $O\underline{C}H_2Ph$), 74.8 (d, C_6), 127.9, 128.4, and 136.0 (aromatic carbons), 155.3 (s, NHCO), 169.3 (s), 170.8 (s). *Anal.* Calcd for $C_{16}H_{19}NO_6$: C, 59.81; H, 5.96; N, 4.36. Found: C, 59.88; H, 6.01; N, 4.26.

(3*S*,4*R*)-3-[(*R*)-1-Hydroxyethyl]-4-[(benzyloxycarbonyl)methyl]-2-azetidinone (19a) **17a** (195 mg, 0.6 mmol) was catalytically hydrogenated with 5% Pd–C (75 mg) in MeOH (9 ml) for 1 h. After removal of the catalyst, the filtrate was concentrated *in vacuo* to give a residue, to which 3 ml of concentrated HCl was added. The mixture was heated under reflux for 40 min, then the HCl was removed *in vacuo*. After repeated evaporation from benzene, a mixture of the crude hydrochloride of **17b** (126 mg, yield quant.) and benzyl alcohol (0.9 ml) was stirred at 70 °C for 7 h, then allowed to cool to room temperature. Benzyl alcohol (0.9 ml), *N*,*N*′-dicyclohexylcarbodiimide (126 mg, 0.6 mmol) and TEA (0.063 ml, 0.62 mmol) were added, and the reaction mixture was stirred at 55 °C for 5.5 h. Then, the precipitate was removed by filtration. The filtrate was subjected to column chromatography (silica gel, benzene : AcOEt = 1 : 3) to give the β-lactam, **19a** (102 mg, yield 64%) as an oil, $[\alpha]_D^{20}$ +9.9° (c=2.3, $CHCl_3$), (lit.[6g]) $[\alpha]_D^{20}$ +9.84° (c=2.1, $CHCl_3$)). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3305, 1755. ^{1}H-NMR ($CDCl_3$): 1.27 (3H, d, J=6.3 Hz, CH_3), 2.74 (2H, d, J=6.8 Hz, $C\underline{H}_2COOBn$), 2.86 (1H, dd, J=2.2, 6.6 Hz, C_3-H), 3.09 (1H, brs, OH), 3.94 (1H, dt, J=2.2, 6.8 Hz, C_4-H), 4.14 (1H, dq, J=6.3, 6.6 Hz, $CH_3C\underline{H}$), 5.13 (2H, s, $C\underline{H}_2Ph$), 6.52 (1H, brs, NH), 7.35 (5H, s, aromatic protons). ^{13}C-NMR ($CDCl_3$): 21.3 (q, CH_3), 39.4 (t, $\underline{C}H_2COO$), 47.6 (d, C_4), 64.0 (d, C_3), 65.3 (d, $CH_3\underline{C}H$), 66.9 (t, CH_2Ph), 128.4, 129.1, and 135.3 (aromatic carbons), 168.0 (s), 171.2 (s). MS m/z: 263 (M^+).

(3*S*,4*R*)-3-[(*R*)-1-Hydroxyethyl]-4-[3-(*p*-nitrobenzyloxycarbonyl)-2-oxopropyl]-2-azetidinone (20a) A solution of **19a** (85 mg, 0.325 mmol) in MeOH (2.5 ml) was catalytically hydrogenated using 5% Pd–C (25 mg) at room temperature for 1 h. After removal of the catalyst, the filtrate was concentrated *in vacuo* to afford **19b**, which was dissolved in DMF (0.25 ml) and CH_3CN (1 ml), then *N*,*N*′-carbonyldiimidazole (55 mg, 0.34 mmol) was added. After stirring of the mixture at room temperature for 1.5 h, the magnesium salt of *p*-nitrobenzyl hydrogen malonate (97.5 mg, 0.37 mmol) was added, then the whole was stirred at room temperature overnight. After addition of AcOEt (10 ml) and water (5 ml), the mixture was acidified (pH 2) with 2 N aqueous HCl, followed by extraction with AcOEt (×5). The combined organic extracts were washed with saturated aqueous NaCl and saturated aqueous $NaHCO_3$. The aqueous washings were back-washed with AcOEt. The combined organic solutions were washed with saturated aqueous NaCl. Drying followed by evaporation gave a residue, which was purified by column chromatography to give **20a** (83.8 mg, yield 74%) as crystals, mp 126—127.5 °C (AcOEt), $[\alpha]_D^{20}$ +25.7° (c=1.13, MeOH), (lit.[6b]) mp 121 °C, $[\alpha]_D^{22}$ +21.3° (c=0.31, $CHCl_3$)). IR ν_{max}^{KBr} cm^{-1}: 3420, 3315, 1758, 1727, 1710. ^{1}H-NMR ($CDCl_3$): 1.31 (3H, d, J=6.3 Hz, CH_3), 2.29 (1H, d, J=4.1 Hz, OH), 2.83 (1H, dd, J=2.3, 7.1 Hz, C_3-H), centered at 2.96 (2H, ABX, J=5.6, 7.7 Hz, $CHC\underline{H}_2CO$), 3.59 (2H, s, $COCH_2COO$), 3.95 (1H, ABX, C_4-H), 4.16 (1H, ddq, J=4.1, 6.3, 7.0 Hz, $C\underline{H}CH_3$), 5.27 (2H, s, $COOC\underline{H}_2$), 6.03 (1H, brs, NH), 7.53 (2H, d, J=9 Hz, aromatic protons), 8.24 (2H, d, J=9 Hz, aromatic protons). High-resolution MS m/z ($(M+1)^+$): Calcd for $C_{16}H_{19}N_2O_7$: 351.1508. Found: 351.1148.

***p*-Nitrobenzyl (3*R*,5*R*,6*S*)-6-[(*R*)-1-Hydroxyethyl]-1-carba-2-oxopenam-3-carboxylate (21)** A mixture of **20a** (80 mg, 0.22 mmol), *p*-carboxybenzenesulfonyl azide (58 mg, 0.25 mmol), and TEA (0.1 ml, 0.72 mmol) in AcOEt (0.8 ml) was stirred at room temperature for 30 min. After addition of H_2O, the precipitate was collected, and washed with H_2O and ether. Drying over P_2O_5 *in vacuo* gave the diazo derivative (**20b**) (63.6 mg, yield 74%) as a pale yellow powder, mp 157—159.5 °C, $[\alpha]_D^{20}$ +39.6° (c=0.56, MeOH), (lit.[17]) mp 151—152 °C, $[\alpha]_D^{22}$ +21.8° (c=0.23, CH_3CN)). IR ν_{max}^{KBr} cm^{-1}: 3390, 2130, 1736, 1708, 1657. ^{1}H-NMR ($CDCl_3$): 1.33 (3H, d, J=6.4 Hz, CH_3), 2.58 (1H, brs, OH), 2.58 (1H, dd, J=2.2, 7.6 Hz, C_3-H), centered at 3.27 (2H, ABX, J=6.1, 7.3, 18.1 Hz, $CHC\underline{H}_2CO$), 3.98 (1H, ABX, C_4-H), 4.18 (1H, dq, J=6.1, 6.7 Hz, $C\underline{H}CH_3$), 5.36 (2H, s, $COOCH_2$), 5.96 (1H, brs, NH), 7.54 (2H, d, J=9 Hz, aromatic protons), 8.24 (2H, d, J=9 Hz, aromatic protons). MS m/z: 330 $[(M-(N_2 \text{ and } OH))^+]$. A solution of **20b** (56 mg, 0.15 mmol) and $Rh_2(OAc)_4$ (3 mg) in benzene (2 ml) was heated under reflux for 3 min, then allowed to cool to room temperature. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to give **21** (38.5 mg, yield 74%) as crystals, mp 104—106 °C, $[\alpha]_D^{20}$ +155.6° (c=0.46, CH_3CN), (lit.[18]) mp 110—111 °C). IR ν_{max}^{KBr} cm^{-1}: 3460, 3400, 1749. ^{1}H-NMR ($CDCl_3$): 1.41 (3H, d, J=6 Hz, CH_3), 1.78 (1H, d, J=5 Hz, OH), 2.46 (1H, dd, J=8, 19 Hz, C_1-H_a), 1.97 (1H, dd, J=7, 19 Hz, C_1-H_b), 3.21 (1H, dd, J=2.5, 7 Hz, C_6-H), 4.17 (1H, ddd, J=2.5, 7 Hz, C_5-H), 4.32 (1H, dq, J=6, 7 Hz, $CH_3C\underline{H}$), 4.77 (1H, s, C_3-H), 5.30 (2H, s, $COOCH_2$), 7.52 (2H, d, J=8 Hz, aromatic protons), 8.24 (2H, d, J=8 Hz, aromatic protons). High-resolution MS m/z (M^+): Calcd for $C_{16}H_{16}N_2O_7$: 348.0955. Found: 348.0927.

***p*-Nitrobenzyl (5*R*,6*S*)-6-[(*R*)-1-Hydroxyethyl]-2-[(*p*-nitrobenzyloxycarbonyl)aminoethylthio]-1-carbapen-2-em-3-carboxylate (22)** A solution of *N*,*N*-diisopropylethylamine (13 mg, 0.1 mmol) in CH_3CN (0.2 ml) was added to a solution of **21** (30 mg, 0.086 mmol) in CH_3CN (0.75 ml) at −20 °C over a period of 2 min, then a solution of diphenyl phosphorochloridate (26 mg, 0.095 mmol) in CH_3CN (0.2 ml) was added at −20 °C over a period of 2 min. The mixture was stirred at −20 °C for 1.5 h, then *N*-*p*-nitrobenzyloxycarbonylaminoethanthiol (24.3 mg, 0.095 mmol) was added and stirring was continued at −20 °C for 2.5 h. After addition of ether (3 ml), the precipitate was collected and dried *in vacuo* to afford **22** (32.7 mg, yield 65%) as a powder, mp 173.5—175 °C, $[\alpha]_D^{20}$ +59.5° (c=0.3, DMF), (lit.[18]) mp 167—169 °C). IR ν_{max}^{KBr} cm^{-1}:

3410, 3300, 1775, 1690. ^{1}H-NMR ($CDCl_3$): 1.26 (3H, d, J=6 Hz, CH_3), 3.08 (2H, m, SCH_2), 3.32 (1H, dd, J=2, 6 Hz, C_6-H), 3.20—3.60 (4H, m, CH_2N, C_1-H_{ab}), 4.12 (1H, dq, J=6, 6.5 Hz, $CH_3C\underline{H}$), 4.27 (1H, ABX, C_5-H), 5.24 (2H, s, $C\underline{H}_2Ph$), 5.30 and 5.55 (2H, AB, J=13.8 Hz, $C\underline{H}_2Ph$), 6.92 (1H, br s, NH), 7.66, 7.83 and 8.21 (4×2H, each d, J=8.5 Hz, aromatic protons). MS m/z: 581 ($(M+1)^+$).

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Self-Association of Telomeric Short Oligodeoxyribonucleotides Containing a dG Cluster

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Received February 12, 1991

Oligonucleotides containing a dG cluster, $d(T_mG_nT_m)$, are models of single-stranded parts of telomeric deoxyribonucleic acid and substitutes for poly(dG). Electrophoretic and spectroscopic analyses of the oligomers indicate that the oligomers can form two alternative structures, single- and quadruple-stranded helices, in solution at room temperature. The transformation of the single-stranded form into the quadruple-stranded form or *vice versa* is undetectable in 0.1 M NaCl at 4.3×10^{-5} M strand concentration at room temperature. However, at a 50-fold higher strand concentration, the single-stranded oligomer is gradually converted into the quadruplex. An increase in ionic strength stabilizes the single-stranded structure, so it seems to inhibit the formation of the quadruplex. The quadruplex, $[d(TTGGGGTT)]_4$, is resistant to denaturation in 7 M urea, in which the Watson–Crick type d(TTGGGGTT)·d(AACCCCAA) duplex dissociates. An increase in the number of T residues facilitates the dissociation of the quadruplex by heating. Thus the number of T residues surrounding the dG cluster might control the rigidity of the quadruplex structure.

Keywords oligodeoxyribonucleotide; dG cluster; quadruplex; polyacrylamide gel electrophoresis; CD

Guanine-rich sequences are found in many locations on chromosomes such as in telomeres, which are the structures at the ends of eukaryotic chromosomes. Telomeric deoxyribonucleic acid (DNA) is composed of a dC-rich strand and a complementary dG-rich strand that is made up of a large number of repetitive dG cluster sequences,[1] with a single-stranded 3′-terminal overhang of two more dG cluster units.[2] The consensus repeating unit of the dG-rich cluster is $d(T_{1-3}(T/A)G_{3-4})$. In the case of *Tetrahymena* and *Oxytricha*, the sequences of single-stranded dG-rich overhangs are repeated $d(TTGGGG)_2$ and $d(TTTTGGGG)_2$, respectively.[2a,3] These significant dG-rich tails appear to form unusual structures and to be available for telomere maintenance and replication.[1] Telomeric DNA is resistant to nuclease.[4]

Oligonucleotides rich in guanine tend to aggregate and the aggregates of guanine derivatives and 5′-guanosine monophosphate (5′-GMP) form gels at high salt concentrations due to tetrameric molecular arrangement.[5] This arrangement is reproducible in the parallel quadruple-stranded helices of poly(G) and its 2-deamino analogue poly(I).[6] However, because of the heterogeneity of aggregates of guanine-rich oligomers and the improbability of a biological role for the quadruple helix, detailed structural study of the quadruplex has not been done. In particular, very little structural information is available for the deoxyribonucleotide poly(dG). With respect to telomeric oligonucleotide analogues, their structural properties have been examined principally by native gel electrophoresis and by chemical modifications.[7] The formation of parallel[7a,d] and antiparallel[7b,c] quadruple helices by intra- and intermolecular interactions has been proposed. Parallel quadruplex formation during meiotic prophase at physiological salt conditions has been suggested not only in the telomere regions but also in the dG cluster regions such as the immunoglobulin switch regions.[7a]

To elucidate structural properties of helices composed of dG clusters in solutions, we chose the simplified oligodeoxyribonucleotide model, $d(T_mG_nT_m)$, which is made up of a dG cluster and two dT clusters surrounding it. Here we report the properties of a series of telomeric short oligodeoxyribonucleotides as evaluated by polyacrylamide gel electrophoresis and ultraviolet (UV) and circular dichroism (CD) spectroscopies. The octamer d(TTGGGGTT) from single-stranded regions of *Oxytricha* and *Tetrahymena* telomeres was examined as a standard for these model compounds.

Results and Discussion

Polyacrylamide Gel Electrophoresis Self-association of the oligonucleotides, synthesized by the triester method,[8] containing a dG cluster was observed in native and denaturing 25% polyacrylamide gels (Fig. 1). The mobility of the denatured octamer d(TTGGGGTT) was almost identical to that of the single-stranded size marker $d(T_8)$ (Fig. 1A, lanes 1 and 2). In contrast, d(TTGGGGTT) stored at −20 °C (10 A_{max} units/ml) after heat denaturation migrated more slowly than the heat-denatured octamer as a single band (Fig. 1A, lane 3). This band interacted with ethidium bromide, an intercalator (Fig. 1A, lane 6), and the

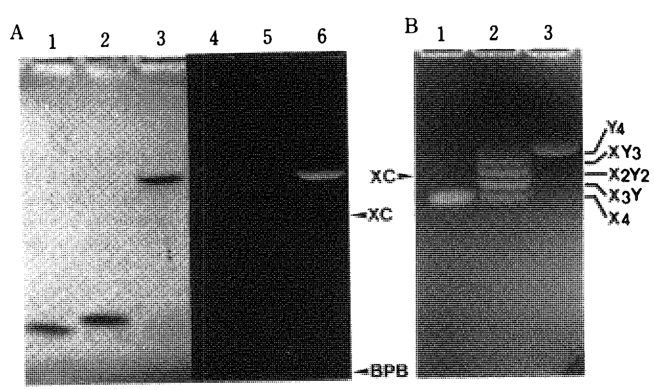

Fig. 1. Demonstrations of Self-Association of d(TTGGGGTT) (A) and Stoichiometry of Self-Associated Oligonucleotides (B)

A: Denaturing (7 M urea) 25% polyacrylamide gel electrophoresis was done at room temperature in 0.4×TBE. Lanes 1 and 4, $d(T_8)$; lanes 2 and 5, d(TTGGGGTT) completely denatured by heating at 95 °C for 5 min; lanes 3 and 6, d(TTGGGGTT), maintained at −20 °C for a few days after heat-denaturation. DNA bands were made visible by UV shadowing (lanes 1 to 3) and ethidium bromide staining (lanes 4 to 6). XC and BPB indicate xylene cyanol and bromophenol blue, respectively. B: Native 25% polyacrylamide gel electrophoresis was done at room temperature in 0.4×TBE. Lane 1, self-associated octamer, $[d(TTGGGGTT)]_4$, (X_4); lane 2, mixture of aggregates of d(TTGGGGTT), (X), and d(TTTGGGGTTT), (Y); lane 3, self-associated decamer, $[d(TTTGGGGTTT)]_4$, (Y_4). Co-annealing of single-stranded X and Y was done by maintaining an equimolecular mixture at −20 °C. DNA bands were detected by ethidium bromide staining.

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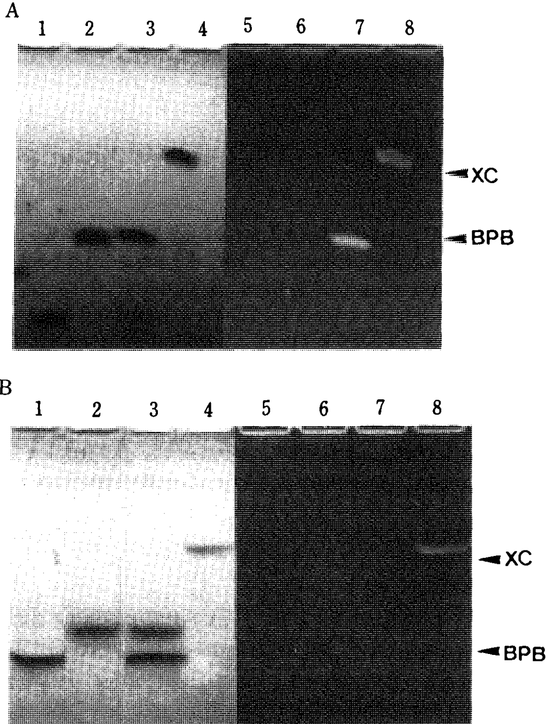

Fig. 2. Electrophoresis of Octamers in Native (A) and Denaturing (B) Polyacrylamide Gels

Lanes 1 and 5, d(AACCCCAA); lanes 2 and 6, d(TTGGGGTT) denatured as described in Fig. 1; lanes 3 and 7, a mixture of equimolecular amounts of d(TTGGGGTT) and d(AACCCCAA); lanes 4 and 8, $[d(TTGGGGTT)]_4$. Lanes 1 to 4, UV shadowing; lanes 5 to 8, ethidium bromide staining.

TABLE I. Electrophoretic Properties of the Oligomers[a)]

Compound	Relative mobility (Rm)[b)]	
	S[c)]	Q[c)]
d(TTGGGGTT)	0.81	0.38
d(TTGGGGGTT)	0.79	0.33
d(TTGGGGGGTT)	0.76	0.30
d(TGGGGT)	0.87	0.50
d(TTTGGGGTTT)	0.75	0.27
d(TTAGGGTT)	0.85	ND[d)]
d(AACCCCAA)	0.94	
d(TTGGGGTT) d(AACCCCAA)		0.99[e)]

a) 7 M urea–25% polyacrylamide gel electrophoresis was done at room temperature (about 23 °C) in 0.4 × TBE (pH 8.3). *b)* With respect to marker dye BPB (1.00). *c)* S, single-stranded helix; Q, quadruple-stranded helix. *d)* ND: not detected. The quadruplex was not detected under either native or denaturing conditions. *e)* The value is the mobility of the duplex, run through native 25% polyacrylamide gel at 4 °C.

position of the band corresponded to that of a single-stranded 30—40 mer.[9)] This phenomenon was reproducible on a native gel and was also observed for the other dG-rich oligomers listed in Table I, with the exception of the octamer, d(TTAGGGTT), which is found in human chromosomal telomeres.[5a)] These data suggest that these dG-rich oligomers spontaneously aggregate to generate a single complex during storage at −20 °C, and that no transformation between the single helix and the complex occurs during gel electrophoresis at room temperature.

To measure the stoichiometry of the self-associated dG-rich oligomers, two different-sized oligomers containing the same dG cluster were co-annealed to form complexes.

TABLE II. UV Absorption Data for the Oligomers[a)]

Compound	Helix type[b)]	λ_{max} (nm)	ε[c)]	Hypochromicity (%)[d)]
d(TTGGGGTT)[e)]	S	256	9500	15
	Q	257	8800	19
d(TTGGGGGTT)	S	256	9500	15
	Q	256	9100	19
d(TTGGGGGGTT)	S	256	10000	15
	Q	256	9200	19
d(TGGGGT)	S	254	9200	21
	Q	255	9600	20
d(TTTGGGGTTT)	S	258	8700	15
	Q	259	8600	16
d(TTAGGGTT)	S	255	9100	22

a) UV spectra of the oligomers (about 1 A_{max} unit/ml) were measured at room temperature (about 23 °C) in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). *b)* The procedure of the sample preparation is described in Experimental. S, single-stranded helix; Q, quadruplex. *c)* Molar absorption coefficient per base residue at λ_{max}. *d)* Absorbance at 260 nm before and after nuclease P1 digestions were compared. *e)* The UV spectra are shown in Fig. 3.

Figure 1B shows co-annealing of d(TTGGGGTT), X, and d(TTTGGGGTTT), Y. If the complexes were quadruplexes, five distinct bands, corresponding to X_4, X_3Y, X_2Y_2, XY_3, and Y_4, should be detected in the gel. The predicted bands were detected (Fig. 1B). Therefore, these complexes are self-associated, intermolecular, quadruple helices.

To compare the stability of the quadruplex, $[d(TTGGGGTT)]_4$, with that of the normal duplex, d(TTGGGGTT)·d(AACCCCAA), native and denaturing gel electrophoreses were done in 25% polyacrylamide gels at 4 °C (Figs. 2A and 2B, respectively). A quadruplex sample and a mixture of the equivalent amount of complementary single-stranded oligomers were applied to the gel. The quadruplex retained its conformation under both electrophoretic conditions. On the other hand, the equimolar mixture of complementary oligomers formed duplexes under the native conditions (Fig. 2A, lanes 3 and 7), and the duplex dissociated under the denaturing conditions (Fig. 2B, lanes 3 and 7). A single-stranded dG-rich oligomer, d(TTGGGGTT), formed a duplex preferentially in the presence of the complementary oligomer (Fig. 2A, lane 7), and no quadruplex was formed even during storage of the mixture at −20 °C.

In contrast to the C2′-endo sugar puckering of B-DNA, the sugar puckering of the quadruple helix of poly(G) is C3′-endo.[6a)] Further, taking into account the differences in rotations per nucleotide and the distances from the helix axis to the sugar-phosphate backbone between duplexes and quadruplexes, the sugar-phosphate backbone of the quadruple helix seems to be stretched considerably. This distortion may make the formation of the quadruplex difficult. On the other hand, the quadruplex may be stabilized by the stacking of Hoogsteen hydrogen-bonded guanine tetrads and by the lack of a major groove, which is exposed to denaturants.

UV Absorption and CD Spectra For quadruplex formation, Na^+ and Rb^+ ions are the most effective of all alkali metal ions.[7d)] Therefore, the oligomers were dissolved in NaCl solution. UV spectral data of the oligomers containing a dG cluster (approximately 5×10^{-5} M strand concentration) are shown in Table II. The UV spectra of the octamer, d(TTGGGGTT), at pH 7 are shown in Fig.

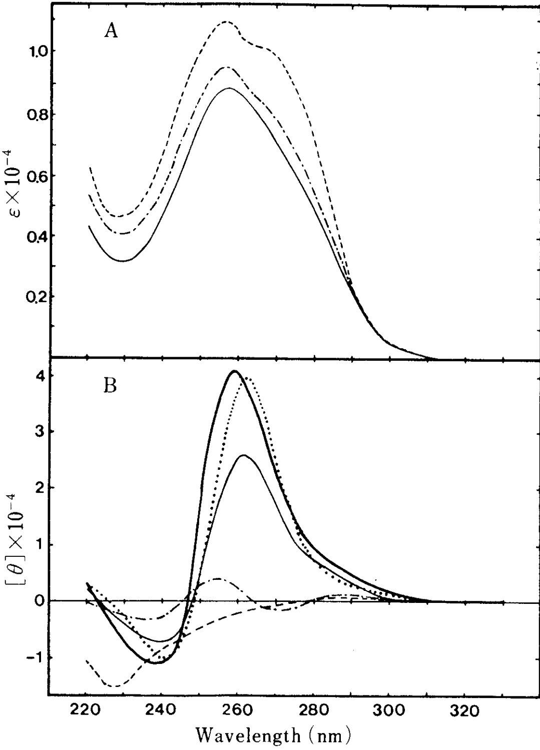

Fig. 3. UV and CD Spectra of d(TTGGGGTT) and Related Compounds

——, $[d(TTGGGGTT)]_4$; —·—, d(TTGGGGTT); – – –, after nuclease PI digestion of d(TTGGGGTT); and ····, $[d(TGGGGT)]_4$ in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0) at 23 °C. ——, poly(G) taken from ref. 10. Oligomer strand concentration: 5×10^{-5} M.

3. The λ_{max}'s of the single helices are about 1 nm shorter in wavelength than those of the quadruplexes. The hypochromicities of the quadruplexes at 260 nm are 4% larger than those of the single helices of d(TTG$_n$TT), but, the differences between single helices and quadruplexes are small for d(TGGGGT) and d(TTTGGGGTTT). The changes of UV spectral shapes, particularly the disappearance of the shoulder around 263 nm, accompanying the transformation of the oligomers from single helices to quadruplexes, are common phenomena for all oligomers that form quadruplexes. The spectral differences between single helices and quadruplexes may be due to differences in the mode and strength of their base–base interactions.

CD spectral data of the oligomers containing a dG cluster are shown in Table III. The CD spectra of the single and the quadruple helices of d(TTGGGGTT) and the quadruplex of d(TGGGGT) are shown in Fig. 3. The single-stranded oligomers have small CD bands, positive bands at around 285 and 255 nm and negative bands at around 270 and 235 nm. In contrast, the spectra of the quadruplexes are very different from those of the single helices, especially in the large positive bands for the quadruplexes at around 260 nm. The difference in CD spectra between single helices and quadruplexes is much larger than that of the UV spectra. Thus the rigid quadruple-stranded structure may favor the induction of CD bands. The spectra of the quadruplexes, especially of d(TGGGGT), are very similar to that of poly(G),[10] which has been assumed to take a parallel quadruple-stranded structure. It seems reasonable to assume that these model complexes adopt parallel quadruple-stranded structures like that of poly(G).

TABLE III. CD Data for the Oligomers[a]

Compound	Helix type[b]		λ_{max} (nm)	($[\theta] \times 10^{-3}$)	
d(TTGGGGTT)[c]	S	286 (1.4)	269 (−1.3)	254 (4.0)	237 (− 3.7)
	Q		263 (27.1)		242 (−10.4)
d(TTGGGGGTT)	S	288 (1.0)	272 (−1.8)	254 (4.0)	236 (− 3.4)
	Q		262 (30.1)		242 (−10.9)
d(TTGGGGGGTT)	S		274 (−3.1)	256 (10.8)	236 (− 5.2)
	Q		262 (34.2)		242 (−11.8)
d(TGGGGT)	S		273 (−4.3)	254 (11.0)	235 (− 3.5)
	Q		262 (38.2)		241 (−11.3)
d(TTTGGGGTTT)	S	282 (3.1)	266 (2.5)	256 (2.5)	237 (− 3.2)
	Q		264 (18.5)		241 (−11.3)
d(TTAGGGTT)	S			256 (4.8)	238 (− 3.6)

a) Immediately after sample preparation, CD spectra of the oligomers (about 1 A_{max} unit/ml) were measured at room temperature in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0). *b*) S, single-stranded helix; Q, quadruplex. *c*) The CD spectra are shown in Fig. 3.

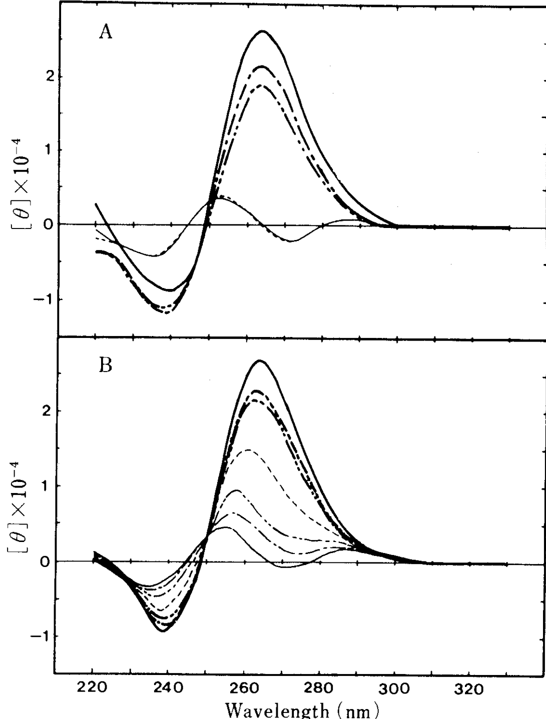

Fig. 4. CD Spectra of Quadruple- and Single-Stranded d(TTGGGGTT) after Various Incubation Times

Measured in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.0) at a strand concentration of 5×10^{-5} M (A) or 1×10^{-3} M (B) at room temperature (about 23 °C). Single-stranded octamer: ——, immediately after preparation; —·—, 5 d; —·-·—, 10 d; – – –, 40 d. Quadruple-stranded octamer: ——, immediately after preparation; —·—, 5 d; —·-·—, 12 d.

Progressive spontaneous change of the structure of the octamer, d(TTGGGGTT), is observed with the lapse of time. When the strand concentration was approximately 4×10^{-5} (Fig. 4A), the spectra of the heat-denatured single-stranded octamer showed no change with time over a period of about 40 d. But, in the case of the octamer quadruplex, the $[\theta]$ value decreased gradually. This is not due to the transformation of the structure from quadruplex to single helix, because no single helix band was detected by polyacrylamide gel electrophoresis for a quadruplex sample maintained for 12 d at room temperature (data not shown).

Duplex formation of complementary oligomers is accelerated by increasing the oligomer concentrations.[11]

The changes of the CD spectra of both single helix and quadruplex samples, during storage at room temperature, were measured at a 50-fold higher strand concentration (approximately 2×10^{-3} M). The spectral conversion pattern of the quadruplex was almost the same as that of the quadruplex at the low oligomer concentration. The spectral conversion of the single helix to the quadruplex was observed for more than 40 d. There are two isosbestic points at 228 and 250 nm (Fig. 4B). In addition, no bands corresponding to the duplex and the triplex were detected by polyacrylamide gel electrophoresis (data not shown). These phenomena support the hypothesis that the spectral conversion of the single helix sample is due to transformation from the single helix to the quadruplex.

To observe the effects of salt concentration, CD spectra at a lower oligomer concentration were measured. The CD spectra of the single-stranded d(TTGGGGTT) as a function of salt concentration are shown in Fig. 5A. These CD spectra show that the stacked conformation in a single helix is stabilized with an increase in the salt concentration. A single helix in any amount of salt shows no sign of quadruplex formation. In the case of the quadruplex, the change in spectra during storage at room temperature, attributed to the transformation from a metastable quadruplex to a stable quadruplex,[10] is negligible in high salt concentration (data not shown).

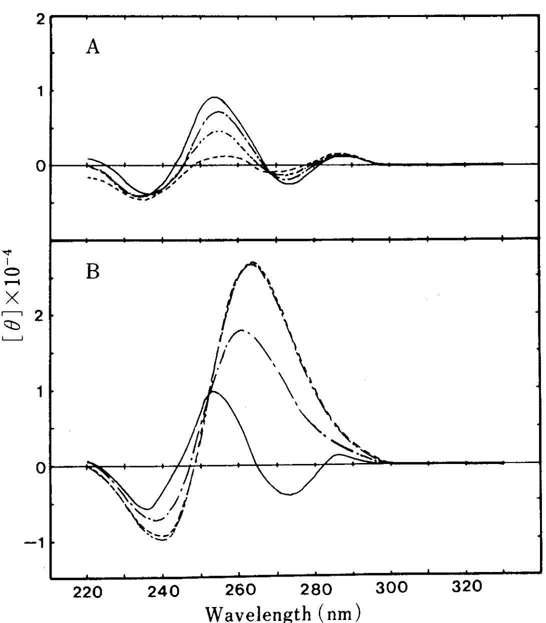

Fig. 5. Influence of Salt Concentration on Structure of Single-Stranded d(TTGGGGTT) (A) and on Transformation from Single Helix to Quadruplex (B)

A: CD spectra immediately after sample preparation in 0.01 M sodium cacodylate (pH 7.0) containing 3.0 M (——), 1.0 M (—·—), 0.1 M (—···—), or 0.01 M NaCl (---) at room temperature (about 23 °C). B: Samples used above (A) were maintained at −20 °C for a few days and then melted at room temperature.

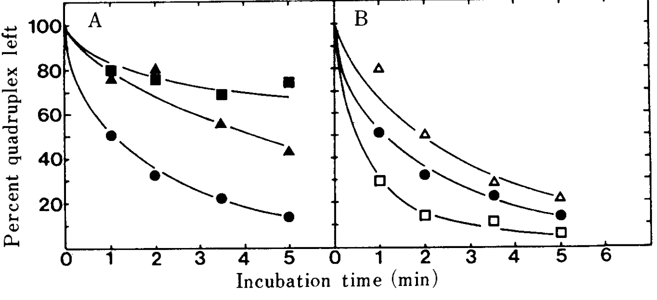

Fig. 6. Course of Dissociation of Quadruplex on Heating

A: Influence of the number of dG residues. ●, [d(TTGGGGTT)]$_4$; ▲, [d(TTGGGGGGTT)]$_4$; ■, [d(TTGGGGGGGTT)]$_4$. B: Influence of the number of T residues. ●, [d(TTGGGGTT)]; △, [d(TGGGGT)]$_4$; □, (TTTGGGGTTT)]$_4$. Samples (0.1 A_{max} units/10 μl) in 0.01 M NaCl–0.01 M sodium cacodylate (pH 7.0) were heated at 65 °C. After cooling of the samples, 25% polyacrylamide gel electrophoresis was done at room temperature.

Figure 5B shows the effects of salt concentration on the formation of the quadruplex during storage of the single-stranded octamer at −20 °C. There were no changes in CD spectra in 3 M NaCl, during storage, but there were marked changes in 0.01 M and 0.1 M NaCl. The increased salt concentration seems to stabilize the base–base interactions of the single-stranded structure and therefore to inhibit the formation of the quadruplex.

Dissociation of the Quadruplex To measure the effects of the number of dG residues and T residues on the stability of the quadruplexes, the course of dissociation of five varieties of quadruplex on heating at 65 °C was measured (Fig. 6). The d(TTGGGGGGGTT) quadruplex, containing the largest number of dG residues, is most strongly resistant to heat denaturation, while the d(TTTGGGGTTT) quadruplex, containing the largest number of T residues, is most readily dissociated to the single helix. This shows that an increase in the number of dG residues stabilizes the quadruplex structure and that an increase in T residues renders the quadruplex structure slightly unstable. The existence of the T cluster may assist dissociation of quadruplexes in the telomere regions.

Conclusions

It has been demonstrated that the structure formed by oligonucleotides containing two or four repeats of the telomeric sequences from *Oxytricha* and *Tetrahymena* is a parallel or an anti-parallel four-stranded helix, in which four guanines are hydrogen-bonded in a square-planar synmetric array.[7] Furthermore, it has been presumed that the formation of parallel quadruple-stranded helices by guanine-rich motifs participates during meiotic prophase.[7a] However, these conclusions were drawn on the basis of data obtained mainly by gel electrophoresis. No detailed study by instrumental analysis has been reported on the structure of these quadruplexes.

In the model systems reported to date, there have been two or more species, which are concatemeric aggregates and the like. The procedure adopted for sample preparations in this study was such that single helices and quadruplexes are formed alternatively in solution at room temperature (Fig. 1A). These helices can not easily transform into each other. Double- and triple-stranded structures,[12] which seem to be denatured states of the poly(G) quadruplex, were not detected in either association or dissociation processes. These properties make it possible to study single and quadruple helices individually. Furthermore, CD spectra suggest that parallel four-stranded structures of ribopolymers, poly(G), are reproducible within self-associated oligodeoxyribonucleotides containing a dG cluster.

In this paper we have shown that the telomeric shoft oligodeoxyribonucleotides containing a dG cluster are

useful for studies on the parallel quadruplex of oligo- and poly(G). This simple and short quadruplex model should be useful to elucidate structural properties of the quadruplex. Our research is presently directed towards more detailed kinetic and thermodynamic studies, which are needed to characterize fully these association and dissociation reactions.

Experimental

Oligonucleotide Synthesis and Purification Oligodeoxyribonucleotides were synthesized by the standard solid-phase phosphotriester method.[8] After the coupling reactions (10 μmol scale), the resin was treated with 1 M tetramethylguanidine *syn-p*-nitrobenzaldoximate in dioxane–water (0.87 : 1.00, v/v) for 48 h and filtered. The filtrate was dried *in vacuo* followed by treatment with concentrated NH_4OH at 60 °C for 6 h. The dimethoxytrityl-containing oligomer was purified on a Sephadex G25 column and on a reverse-phase C_{18} column (Waters). Detritylation was done with 80% AcOH for 15 min at room temperature. The product was purified by DEAE-cellulose (DE52, Whatman) chromatography and reverse-phase high performance liquid chromatography (HPLC) (Shim-pak CLC-ODS, Shimadzu). The purity of the final products was checked by HPLC and was found to be greater than 97%.

Polyacrylamide Gel Electrophoresis Denaturing (7 M urea) and native gel electrophoreses were done in 25% polyacrylamide gels (19 : 1 ratio of acrylamide to bisacrylamide) using 0.4 × TBE buffer (0.36 M Tris–borate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.3). Samples were run at either room temperature or 4 °C with a voltage gradient of 5 V/cm.

DNA sample solutions (2—10 A_{260} units/ml distilled water) were stored at −20 °C and melted at room temperature just before use. Denatured samples were prepared by heating at 95 °C followed by cooling to room temperature, or by incubation in 0.1 mM NaOH for a few minutes followed by neutralization with 0.1 mM HCl. TBE buffer solution was added to the samples to adjust the salt concentration to that of the loading buffer. The samples were then put onto the gel. DNA bands were observed by UV shadowing and ethidium bromide staining. DNA bands were measured with a Shimadzu CS-9000 scanner.

UV Absorption and CD Spectra UV spectra were recorded on a Shimadzu UV-250 spectrophotometer and CD spectra on a Jasco J-600 spectropolarimeter at room temperature (about 23 °C) in 10 and 1 mm light-path cells. Nucleotide concentrations were about 50 and 250 μM. The molar absorption coefficient and the molar ellipticity are presented per base residue. Complete digestion of denatured oligomers with nuclease P1 (5 μg/ml, Seikagaku Kogyo Co.) was done in 0.05 M ammonium acetate (pH 5.3) at 37 °C for several hours.

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Enantiocontrolled Synthesis of the Antifungal β-Lactam (2*R*,5*S*)-2-(Hydroxymethyl)clavam

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The antifungal β-lactam (2*R*,5*S*)-2-(hydroxymethyl)clavam was synthesized in a convergent and stereocontrolled manner from methyl 6-phthalimidopenicillanate and 2,3-*O*-isopropylidene-(*R*)-glyceraldehyde. Convenient methods of acetonide cleavage and phthalimide deblocking are also described.

Keywords (2*R*,5*S*)-2-(hydroxymethyl)clavam; oxapenam; synthesis; antifungal; β-lactam; acetonide; deblocking; phthalimide; diazotization

(3*R*,5*S*)-3-Hydroxymethyl-4-oxa-1-azabicyclo[3.2.0]-heptan-7-one [(2*R*,5*S*)-2-(hydroxymethyl)clavam] (**1**) is an antifungal β-lactam isolated by Brown and Evans[1] from culture fluids of *Streptomyces clavuligerus*. From the viewpoint of stereochemistry, this compound is intriguing[2] in that the absolute configuration of C5 (clavam numbering as shown in A) is *S*, which is different from that of natural β-lactam antibacterial agents, such as penicillin and cephalosporin, whose configuration at the corresponding positions is *R*. Structurally related antifungals, such as (hydroxyethyl)clavam,[3] clavalanine,[4] clavamycins,[5] and valclavam[6] have since been isolated and have been shown to possess the same *S* configuration at C5. Syntheses of some of these compounds have already been accomplished. Among them, it is noteworthy that Müller *et al.*,[4c] Bernardo *et al.*,[7] and Hoppe *et al.*[8] have successfully achieved the syntheses of these compounds in optically active form. In these studies, however, the stereochemistry, especially at C5, was not perfectly controlled. Recently, clavaminic acid with the 5*S* configuration has been shown to take part in the biosynthesis of clavulanic acid whose configuration at C5 is *R*.[9] The importance of controlling the stereochemistry at this position in the synthesis of these antibiotics prompted us to report here our stereocontrolled synthesis of the title compound **1**.

We planned to utilize monocyclic β-lactams derived from 6-aminopenicillanic acid (6-APA) as intermediates, so the logical strategy to control the stereochemistry at the C5 position was considered to involve C–O bond formation by the attack of an alcohol oxygen on the chiral acylimine (or acyliminium ion) intermediate B bearing a bulky, subsequently removable function Y near the electrophilic center.[10,11] With this prospect in mind, we selected the known phthalimido-acetate **2**[11] as a chiral β-lactam precursor.

A diastereomeric mixture of **3** (racemic form) has already been prepared by Bentley and Hunt.[12] These authors reported that this mixture was separated and that hydrogenolysis of the diastereomer (±)-**3** gave the alcohol (±)-**1**, albeit in a low yield. We therefore decided to synthesize first the benzyl ether **3** in an optically active form, and then to search for the hydrogenolytic conditions that would convert **3** into **1** in a better yield than the literature method. Enantiocontrolled synthesis of **3** was achieved in a manner similar to that detailed below for the synthesis of **4**. However, hydrogenolysis of **3** gave no trace amount of the desired alcohol **1** but gave abnormal ring-cleavage product. The detail of this ring-cleavage reaction will be reported elsewhere.[13]

Consequently, we turned our attention to the *tert*-butyldimethylsilyl ether **4**, and chose a secondary alcohol **5** as the partner of B, since the silyl protecting group might be removed with fluoride anion under nearly neutral conditions.

Methyl 6-phthalimidopenicillanate (**6**),[14] which is readily available from 6-APA, was degraded in two steps into **2** as follows (Chart 2). Thus, heating of **6** in acetic acid in the presence of mercury(II) acetate afforded a monocyclic *trans* β-lactam **7** in 99% yield, and this was cleaved by the catalytic use of potassium permanganate in the presence of excess sodium metaperiodate in phosphate buffer solution, giving **2** in 82% yield. This procedure of preparing **2** was

1 A clavam skeleton B

2 3 : R=CH_2Ph 4 : R=TBDMS 5

Chart 1

6 7 2 Phth=

Chart 2

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Chart 3

found to be better than the literature method[11] in terms of yield and smaller number of steps.

Another segment, the optically active alcohol **5**, was obtained from 2,3-*O*-isopropylidene-(*R*)-glyceraldehyde (**9**) through the following steps (Chart 3). Thus, the addition of sodium borohydride to the reaction mixture containing the aldehyde **9**, obtained by the oxidative cleavage of 1,2:5,6-di-*O*-isopropylidene-D-mannitol (**8**), gave the alcohol **10**. *tert*-Butyldimethylsilylation of **10** in a usual manner gave **11** in 96% overall yield from **8**. Next, it was necessary to cleave selectively the acetonide group in the presence of the acid-sensitive *tert*-butyldimethylsilyl group. Under the ordinary hydrolytic conditions, the silyl group was cleaved faster. However, the report of Kelly *et al.* indicated that the cleavage of a *tert*-butyldimethylsilyl ether in an aprotic solvent needs an excess amount of boron trifluoride etherate.[15] Consequently, **11** was treated in dichloromethane at 0 °C with a catalytic amount of boron trifluoride etherate in the presence of excess 1,3-propanedithiol, affording **12** in 89% yield. Selective tosylation of the primary hydroxyl group in **12** occurred on treatment with *p*-toluenesulfonyl chloride in pyridine at 0 °C, giving **5** in 78% yield.

The alcohol **5** was heated with the acetate **2** in the presence of zinc acetate dihydrate in benzene–toluene with the azeotropic removal of water [12] (Chart 4). Only the *trans* lactam **13** was obtained in 63% yield. The next problem to be solved was the removal of the phthalimido group in **13**. Conversion into the amine **15** was first investigated. Attempted dephthaloylation in a usual manner by mixing with hydrazine hydrate in ethanol resulted in destruction of the β-lactam ring. Monitoring of the reaction by thin-layer chromatography (TLC) revealed that the first step from **13** to give the "hemi-hydrazino" intermediate **14** occurred rapidly, within 0.5 h at 0 °C. But the subsequent transformation into the amine **15** and phthaloyl hydrazide took a long time, during which **14** and **15** suffered considerable decomposition. It occurred to us, however, that this second step could be accelerated by an acidic reaction condition, under which the amide moiety in the intermediate **14** might be activated and the ability of **15** to leave **14** might be enhanced by protonation. Addition of acetic acid to the solution of **14** that was obtained above brought about smooth liberation of the amine **15**, which seemed to be stable under this condition. Since the free amine **15** might be unstable, it was subjected to the next reaction without isolation. Treatment of the above reaction mixture with concentrated hydrochloric acid followed by the addition of potassium nitrite below 0 °C gave a chlorinated lactam **16** in 86% overall yield from **13**. The unmasked hydroxyl group in **16** was silylated again to give **17** in 76% yield. Dechlorination of **17** by heating with tributylstannane and a catalytic amount of α,α'-azobisisobutyronitrile (AIBN) in benzene afforded **18** in 87% yield.

Chart 4

The conversion of **18** into **1** was straightforward. The tosyloxy group in **18** was converted into an iodine atom in a usual manner to give **19** in 86% yield, and this was cyclized by treatment with powdered potassium carbonate in *N*,*N'*-dimethylformamide (DMF) at room temperature to give the oxapenam derivative **4** in 90% yield. The yield was higher than that reported in the literature[12] where related bromides, instead of the iodide **19**, was used as the cyclization precursor. Finally, the *tert*-butyldimethylsilyl protecting group was removed in tetrahydrofuran (THF) in the presence of tetrabutylammonium fluoride, which was buffered with acetic acid, liberating the title compound **1**, $[\alpha]_D^{25}$ $-193°$ (c=1.20, $CHCl_3$), as a colorless oil in 94% yield. The infrared (IR) spectrum and the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of this product

were in accord with those of the racemic material reported in the literature.[12)]

Experimental

Melting points are uncorrected. IR spectra were recorded on a JASCO A-102 spectrometer, ^{1}H-NMR spectra on a Varian EM-360L spectrometer (60 MHz) using tetramethylsilane as the internal standard, and mass spectra (MS) and high-resolution mass spectra (HRMS) on a JEOL JMS D300 spectrometer. Rotations were determined on a Perkin-Elmer 141 spectrometer at 25 °C. TLC was performed on TLC plates, Silica gel $60F_{254}$ precoated, layer thickness 0.25 mm (E. Merck), and spots were made visible by ultraviolet (UV) irradiation or by spraying with phosphomolybdic acid or with vanadic acid-sulfuric acid followed by heating. Preparative TLC was performed on TLC plates, Silica gel $60F_{254}$ precoated, layer thickness 2 mm (E. Merck). Chromatography columns were prepared with silica gel (60—110 mesh, Kanto Chemical Co., Inc.), and flash chromatography columns with silica gel (230—400 mesh, E. Merck). The amount of silica gel used and the developing solvents are shown in parentheses. The abbreviations used are as follows: s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; br, broad.

(3*S*,4*S*)-4-Acetoxy-1-(1-methoxycarbonyl-2-methyl-1-propenyl)-3-phthalimido-2-azetidinone (7) A mixture of methyl (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-phthalimido-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [methyl (3*S*,5*R*,6*R*)-6-phthalimidopenicillanate] (6)[13)] (63.8 g, 177 mmol) and mercury(II) acetate (113.0 g, 355 mmol) in AcOH (700 ml) was heated at 90 °C for 30 min. After cooling, the mixture was diluted with PhH and the precipitates were filtered off. The filtrate was evaporated and the residue was partitioned between AcOEt and water. The organic layer was washed several times with aqueous $NaHCO_3$ until the aqueous layer became basic. Then the organic layer was suction-filtered to remove the precipitates that had appeared in the separatory funnel. The filtrate was washed with brine, dried over Na_2SO_4, and the solvent was evaporated off to give **7** (72.1 g, quantitative yield) as a foam. The IR and ^{1}H-NMR spectra of this product were in accord with those reported in the literature.[11)]

(3*S*,4*S*)-4-Acetoxy-3-phthalimido-2-azetidinone (2) A solution of **7** (24 g, 62 mmol) in acetone (200 ml) was added to an ice-cooled solution of Na_2HPO_4 (14.91 g, 105 mmol), $NaH_2PO_4 \cdot H_2O$ (9.36 g, 68 mmol), $NaIO_4$ (67.2 g, 314 mmol), and $KMnO_4$ (1.3 g, 8 mmol) in water (1150 ml) over a period of 15 min at 0 °C with stirring. Then the solution was diluted with acetone (400 ml). After stirring had been continued at 0 °C for 40 min, the pinkish color of the reaction mixture gradually disappeared. Therefore additional $KMnO_4$ (0.3 g, 2 mmol) was added. After being stirred at 0 °C for an additional 2 h, the mixture was filtered and the precipitates were washed with AcOEt and water. The filtrate and the washings were combined and the organic layer was washed with brine. The extract was dried over Na_2SO_4, and the solvent was evaporated off to give a solid residue, which was recrystallized from AcOEt–hexane to give **2** (14 g, 82%) as a colorless powder, mp 184—188 °C (dec.). The IR and ^{1}H-NMR spectra of this product were in accord with those reported in the literature.[11)]

(*S*)-(2,2-Dimethyl-1,3-dioxolan-4-yl)methanol (10) A solution of $NaIO_4$ (14.15 g, 66 mmol) in water (10 ml) was added to an ice-cooled solution of 1,2:3,4-di-*O*-isopropylidene-D-mannitol (**8**) (11.96 g, 46 mmol) in water (175 ml). After being stirred under ice-cooling for 10 min, the mixture was diluted with EtOH (600 ml) and filtered. The precipitates were washed with EtOH (200 ml), and the filtrate and the washings were combined. The precipitates that emerged were again filtered off. This filtrate, containing 2,3-*O*-isopropylidene-(*R*)-glyceraldehyde (**9**), was treated with $NaBH_4$ (3.38 g, 99 mmol) under ice-cooling, and the mixture was stirred at 0 °C for 1.4 h. The precipitates that emerged were filtered off and the filtrate was concentrated to *ca.* 200 ml. To this was added $CHCl_3$ (200 ml), and the mixture was adjusted to pH 8 by the careful addition of AcOH at 0 °C with stirring. After 10 min, the mixture was extracted 6 times with $CHCl_3$. The extracts were dried over Na_2SO_4 and the solvent was evaporated off, to give **10** (12 g, quantitative yield) as an oil, which was used for the next reaction without further purification, since **10** was found to be prone to racemize *via* acetal migration.

(*R*)-4-(*tert*-Butyldimethylsilyl)oxymethyl-2,2-dimethyl-1,3-dioxolane (11) A mixture of **10** (12 g) obtained above, Et_3N (11.93 g, 118 mmol), and *tert*-butylchlorodimethylsilane (15.1 g, 100 mmol) in DMF (115 ml) was stirred at room temperature for 1.5 h, then cooled with an ice bath. Water was added and the mixture was extracted with PhH. The extract was washed successively with water twice and with brine once, and dried over Na_2SO_4. The concentrated mixture was purified with a flash chromatography column (150 g, PhH) to afford **11** (21.49 g, 96% overall yield from **8**) as an oil, $[\alpha]_D^{25}$ +19.9° (c=1.33, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{26}O_3Si$: C, 58.49; H, 10.63. Found: C, 58.55; H, 10.53. IR ($CHCl_3$): 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.89 (9H, s), 1.35 (3H, s), 1.40 (3H, s), 3.4—4.3 (5H, m). MS m/z: 231 ($M^+ - CH_3$), 189, 171, 131 (100%).

(*R*)-3-(*tert*-Butyldimethylsilyl)oxy-1,2-propanediol (12) $BF_3 \cdot OEt_2$ (90 mg, 0.65 mmol) was added to a mixture of **11** (2.2 g, 8.9 mmol) and 1,3-propanedithiol (2.3 ml, 23 mmol) in CH_2Cl_2 (40 ml) at 0 °C with stirring. After 40 min, a diluted aqueous solution of $NaHCO_3$ was added, and the mixture was extracted with AcOEt twice. The combined extracts were dried over Na_2SO_4 and the solvent was evaporated. The residue was chromatographed on silica gel (50 g, AcOEt : hexane = 3 : 2, v/v) to afford **12** (1.58 g, 89%) as an oil, $[\alpha]_D^{25}$ −0.6° (c=1.31, $CHCl_3$). IR ($CHCl_3$): 3580, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (3H, s), 0.08 (3H, s), 0.90 (6H, s), 3.5—3.9 (5H, m). MS m/z: 207 ($M^+ + 1$), 175, 149, 131 (100%). HRMS Calcd for $C_9H_{23}O_3Si$ ($M^+ + 1$): 207.1419. Found: 207.1417.

(*S*)-1-(*tert*-Butyldimethylsilyl)oxy-3-(*p*-toluenesulfonyl)oxy-2-propanol (5) A mixture of **12** (11.6 g, 56 mmol) and *p*-toluenesulfonyl chloride (10.8 g, 57 mmol) in pyridine (140 ml) was set aside at 0 °C for two nights. Then the mixture was evaporated *in vacuo* and the residue was partitioned between AcOEt and water. The organic layer was washed with water (×2) and brine (×1), and dried over Na_2SO_4. The solvent was evaporated off to leave an oily residue, which was chromatographed on silica gel (300 g, AcOEt : PhH = 1 : 1, v/v) to afford **5** (15.92 g, 78%) as an oil, $[\alpha]_D^{25}$ +7.7° (c=1.07, $CHCl_3$). IR ($CHCl_3$): 3560, 2930, 1600, 1460, 1360, 1174 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.03 (6H, s), 0.85 (9H, s), 2.44 (3H, s), 3.5—4.2 (5H, m), 7.30 (2H, d, J=9 Hz), 7.80 (2H, d, J=9 Hz). MS m/z: 361 ($M^+ + 1$), 303, 229, 131 (100%). HRMS Calcd for $C_{16}H_{29}O_5SSi$ ($M^+ + 1$): 361.1506. Found: 361.1510.

(3*S*,4*S*)-4-[(*S*)-1-(*tert*-Butyldimethylsilyl)oxymethyl-2-[(*p*-toluenesulfonyl)oxy]ethoxy]-3-phthalimido-2-azetidinone (13) A mixture of **5** (0.90 g, 2.5 mmol), **2** (1.17 g, 4.27 mmol), and zinc acetate dihydrate (330 mg, 1.64 mmol) in PhH (8 ml) and toluene (8 ml) was refluxed for 5.5 h under a Dean–Stark condenser which was packed with molecular sieves 4 A. After cooling, the mixture was partitioned between AcOEt, containing a small volume of acetone, and water. The organic layer was washed with brine, dried with Na_2SO_4, and the solvent was evaporated off. Chromatographic purification on silica gel (40 g, AcOEt : PhH = 1 : 4, v/v) afforded **13** (0.91 g, 63% from **5**) as a foam, $[\alpha]_D^{25}$ −54° (c=0.91, $CHCl_3$). IR ($CHCl_3$): 3340, 1790, 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s), 0.85 (9H, s), 2.46 (3H, s), 3.4—3.9 (3H, m), 3.9—4.2 (2H, m), 5.10 (1H, d, J=1 Hz), 5.58 (1H, d, J=1 Hz), 6.85 (1H, br), 7.0—8.0 (8H, m). MS m/z: 574 (M^+), 517, 474, 449, 335, 316, 229 (100%). HRMS Calcd for $C_{27}H_{34}N_2O_8SSi$: 574.1805. Found: 574.1821.

(3*S*,4*S*)-3-Chloro-4-[(*S*)-1-hydroxymethyl-2-[(*p*-toluenesulfonyl)oxy]ethoxy]-2-azetidinone (16) A solution of hydrazine hydrate (60 mg, 1.2 mmol) in a small volume of EtOH was added to a solution of **13** (301 mg, 0.52 mmol) in EtOH (6 ml) at 0 °C and the mixture was stirred at 0 °C for 40 min. Then 0.60 ml of AcOH was added and the whole was stirred at 0 °C for 10 min, and then at room temperature for 1.4 h, during which time phthaloyl hydrazide precipitated gradually. The reaction mixture was cooled to −50 °C, and 6 ml of concentrated HCl was added. The temperature was raised to −15 °C, and a solution of KNO_2 (140 mg, 1.7 mmol) in a small volume of water was slowly added to the mixture over a period of 2 h at this temperature. The temperature was then raised to 10 °C over a period of 0.5 h, and the mixture was added to AcOEt/ice-water containing 11 g of K_2CO_3. The layers were separated, and the organic layer was washed successively with water and brine and dried over Na_2SO_4. The solvent was evaporated off to leave an oily residue, which was chromatographed on silica gel (4 g, AcOEt : PhH = 1 : 1, v/v) to afford **16** (158 mg, 86%) as an oil, $[\alpha]_D^{25}$ −39.6° (c=1.73, $CHCl_3$). IR ($CHCl_3$): 3420, 1790, 1365, 1180 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (3H, s), 3.5—4.3 (5H, m), 4.45 (1H, br s), 5.05 (1H, s), 7.3 (1H, br), 7.33 (2H, d, J=9 Hz), 7.80 (2H, d, J=9 Hz). MS m/z: 349 (M^+), 314, 257, 229, 173, 155 (100%). HRMS Calcd for $C_{13}H_{16}ClNO_6S$: 349.0386. Found: 349.0367.

(3*S*,4*S*)-4-[(*S*)-1-(*tert*-Butyldimethylsilyl)oxymethyl-2-[(*p*-toluenesulfonyl)oxy]ethoxy]-3-chloro-2-azetidinone (17) A mixture of **16** (158 mg, 0.45 mmol), imidazole (60 mg, 0.87 mmol), and *tert*-butylchlorodimethylsilane (90 mg, 0.60 mmol) in DMF (3 ml) was stirred at room temperature for 20 min. Then the mixture was cooled to 0 °C and water was added. The mixture was extracted with PhH and the extracts were washed with water (×2) and brine (×1), successively. The extracts were dried over

Na_2SO_4, and the solvent was evaporated off, to leave an oily residue, which was chromatographed on silica gel (4 g, AcOEt : PhH = 3 : 17, v/v) to give **17** (160 mg, 76%) as an oil, $[\alpha]_D^{25}$ −32.2° (c = 0.98, $CHCl_3$). IR ($CHCl_3$): 3420, 1790, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s), 0.86 (9H, s), 2.45 (3H, s), 3.5—4.0 (3H, m), 4.0—4.2 (2H, m), 4.42 (1H, br s), 5.06 (1H, s), 6.65 (1H, br), 7.48 (2H, d, J = 8 Hz), 7.84 (2H, d, J = 8 Hz). MS m/z: 406 ($M^+ - C_4H_9$), 363, 330, 303, 229, 131 (100%). HRMS Calcd for $C_{15}H_{21}ClNO_6SSi$ ($M^+ - C_4H_9$): 406.0546. Found: 406.0530.

(*S*)-4-[(*S*)-1-(*tert*-Butyldimethylsilyl)oxymethyl-2-[(*p*-toluenesulfonyl)-oxy]ethoxy]-2-azetidinone (18) A mixture of **17** (160 mg, 0.35 mmol), tributylstannane (200 mg, 0.68 mmol), and α,α'-azobisisobutyronitrile (5.7 mg, 0.035 mmol) in PhH (7 ml) was refluxed for 1.5 h under an atmosphere of argon. Then the solvent was evaporated off, the residue was dissolved in CH_3CN, and solution was washed with hexane five times to remove tributylstannane and tributylchlorostannane. The CH_3CN layer was concentrated to leave an oily residue. This was chromatographed on silica gel (3 g, AcOEt : PhH = 1 : 4, v/v) to give **18** (129 mg, 87%) as an oil, $[\alpha]_D^{25}$ −8.9° (c = 2.89, $CHCl_3$). IR ($CHCl_3$): 3420, 1765, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.90 (9H, s), 2.44 (3H, s), 3.5—4.2 (7H, m), 5.13 (1H, dd, J = 4, 3 Hz), 6.5 (1H, br), 7.33 (2H, d, J = 8 Hz), 7.80 (2H, d, J = 8 Hz). MS m/z: 430 ($M^+ + 1$), 414, 372, 330, 229 (100%). HRMS Calcd for $C_{19}H_{32}NO_6SSi$ ($M^+ + 1$): 430.1718. Found: 430.1740. Concentration of the hexane layer and chromatographic purification on a preparative TLC plate (AcOEt : PhH = 1 : 4, v/v) afforded additional **18** (11 mg), but this was contaminated with tributylchlorostannane.

(*S*)-4-[(*S*)-1-(*tert*-Butyldimethylsilyl)oxymethyl-2-iodoethoxy]-2-azetidinone (19) A mixture of **18** (129 mg, 0.30 mmol) and NaI (0.67 g, 4.5 mmol) in acetone (10 ml) was refluxed in an oil bath for 14 h. The mixture was partitioned between AcOEt and water, and the organic layer was washed with aqueous $NaHCO_3$ solution and brine. After being dried over Na_2SO_4, the mixture was purified by flash chromatography (2 g, AcOEt : PhH = 3 : 17, v/v) to afford **19** (100 mg, 86%) as a solid. Recrystallization from hexane gave an analytical sample, mp 40—42 °C, $[\alpha]_D^{25}$ −18.7° (c = 1.50, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{24}INO_3Si$: C, 37.41; H, 6.28; N, 3.64. Found: C, 37.28; H, 6.32; N, 3.68. IR ($CHCl_3$): 3430, 1770 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.89 (9H, s), 2.9—3.4 (4H, m), 3.4—4.0 (3H, m), 5.20 (1H, dd, J = 4, 2 Hz), 6.5 (1H, br). MS m/z: 328 ($M^+ - C_4H_9$), 299, 284, 260, 255, 241, 144, 70 (100%).

(3*R*,5*S*)-3-(*tert*-Butyldimethylsilyl)oxymethyl-4-oxa-1-azabicyclo[3.2.0]-heptan-7-one (4) A mixture of **19** (164 mg, 0.43 mmol) and powdered K_2CO_3 (147 mg, 1.07 mmol) in DMF (2.1 ml) was stirred at 26 °C for 8 h. Then it was diluted with benzene, and was washed with water (×3), phosphate buffer (pH 7, ×1), and brine (×1), successively. The extracts were dried over Na_2SO_4, the solvent was evaporated off, and the residue was chromatographed on silica gel (2 g, AcOEt : hexane = 1 : 19, v/v) to afford **4** (98 mg, 90%) as an oil, $[\alpha]_D^{25}$ −104° (c = 1.68, $CHCl_3$). IR ($CHCl_3$): 2930, 1770, 1460, 1330, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.90 (9H, s), 2.77 (1H, d, J = 16 Hz), 2.92 (1H, dd, J = 11, 5 Hz), 3.22 (1H, d, J = 16, 3 Hz), 3.5—3.8 (2H, m), 3.89 (1H, dd, J = 11, 7 Hz), 4.2—4.6 (1H, m), 5.31 (1H, d, J = 3 Hz). MS m/z: 258 ($M^+ + 1$), 242, 216, 200, 158, 131 (100%). HRMS Calcd for $C_{12}H_{24}NO_3Si$ ($M^+ + 1$): 258.1526. Found: 258.1510.

(3*R*,5*S*)-3-Hydroxymethyl-4-oxa-1-azabicyclo[3.2.0]heptan-7-one [(2*R*,5*S*)-2-(Hydroxymethyl)clavam] (1) A THF solution of 1 N Bu_4NF (1.3 ml, 1.3 mmol) containing AcOH (48 mg, 0.8 mmol) was added to a solution of **4** (167 mg, 0.65 mmol) in THF (2.7 ml). The mixture was stirred at room temperature for 1.5 h, then poured onto a column of silica gel (5 g). The column was washed with 150 ml of AcOEt. The eluates were combined, the solvent was evaporated, and the residue was chromatographed on silica gel (3 g, AcOEt : PhH = 1 : 1, v/v) to give **1** (87 mg, 94%) as an oil, $[\alpha]_D^{25}$ −193° (c = 1.20, $CHCl_3$). MS m/z: 144 ($M^+ + 1$), 115 (100%), 70. HRMS Calcd for $C_6H_{10}NO_3$ ($M^+ + 1$): 144.0660. Found 144.0655. The IR and ^{1}H-NMR spectra were in accord with those of the racemic material reported in the literature.[12]

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Photocyclization of Enamides. XXXIV.[1] A Practical Total Synthesis of Aromatic Yohimboid Alkaloids, Oxogambirtannine and Naucleficine[2]

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Total synthesis of naucleficine (3a) was accomplished for the first time *via* an alkaloid, oxogambirtannine (3b), which was efficiently prepared by photocyclization of the *o*-methoxy-enamide 2b. The method is a practical one, suitable for large-scale preparation.

Keywords naucleficine; oxogambirtannine; alkaloid; total synthesis; photocyclization; enamide

Naucleficine (**3a**) was isolated as a minor alkaloid from the stems of *Nauclea officinalis* (Rubiaceae) which has been used as an anti-inflammatory and anti-bacterial herb in Chinese folk medicine.[3] Since its natural availability is low, large-scale chemical synthesis is desirable for the elucidation of its latent pharmacological activities and possibly also for its practical use. We now report in full detail an efficient and practical synthesis of naucleficine through a route involving enamide photocyclization,[4] which makes the alkaloid readily available by a convenient, practical method that is well suited to a multigram-scale synthesis.

As a preliminary experiment, we first investigated photocyclization of the enamide **2a**,[5] which was readily prepared by the acylation of harmalane with isophthalic monoacid chloride **1a**. The enamide **2a** was so unstable that it was, without purification, subjected to the following photocyclization. Irradiation of the crude enamide **2a** with a high-pressure mercury lamp through a Pyrex filter in either acetonitrile or benzene gave the photocyclized lactam **4a** in 80—81% yield as a sole product. The product **4a** exhibited a molecular ion peak at *m/z* 344 in the mass spectrum (MS) and showed proton nuclear magnetic resonance (^{1}H-NMR) signals due to an olefinic and aromatic protons at δ 7.15 (1H, s, 14-H) and 8.84 (1H, d, J=1.5 Hz, 19-H), respectively. These spectral data suggested its benzindoloquinolizine structure with a methoxycarbonyl group at the 18-position. Thus, photocyclization of the enamide **2a** was proved to proceed exclusively in the undesired direction at the *p*-position of the methoxycarbonyl group.

Previously, we have demonstrated that regioselective photocyclization[6] of an *o*-methoxy-substituted enamide proceeds regioselectively at the root of the *o*-methoxyl group and shown that the introduction of a methoxyl group into an *o*-position of the amido group effectively controls the direction of cyclization. Therefore, we prepared the *o*-methoxy-enamide **2b** and irradiated it in order to prepare the benzindoloquinolizine with a methoxycarbonyl group at the 16-position. The starting 2-methoxyisophthalic acid ester **1c** was prepared as follows. Oxidation of 2-hydroxy-3-methylbenzoic acid with lead dioxide gave 2-hydroxy-isophthalic acid **1b** (68% yield), which was then alkylated with methyl iodide in the presence of potassium carbonate to give dimethyl 2-methoxy-isophthalate **1c** in 76% yield. Acylation of harmalane with the acid chloride **1d**, which was prepared by successive treatment of the ester **1c** with an equivalent amount of potassium hydroxide and then thionyl chloride, gave the desired enamide **2b** in good yield. This enamide **2b** was also unstable and was characterized only by ^{1}H-NMR [δ 5.01 and 5.86 (each 1H, br s, C=CH_2)]; without purification, it was subjected to irradiation in three different solvent systems [benzene, acetonitrile, and ether–methanol (40:1)] to afford a mixture of two photocyclized lactams **3b** and **4b**, which were readily isolated by column chromatography in the yields shown in Table I.

The main product **3b** exhibited a molecular ion peak at

harmalane

	R¹	R²	R³
1a	Cl	H	Me
1b	OH	OH	H
1c	OMe	OMe	Me
1d	Cl	OMe	Me

2a: R= H
2b: R= OMe

3a: R= CHO
3b: R= COOMe
3c: R= CH_2OH

4a: R= H
4b: R= OMe

Chart 1

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TABLE I. Photocyclization of the Enamide **2b**

Solvent	Yield (%)[a]	
	3b	**4b**
MeCN	53	26
C_6H_6	58	19
Et_2O–MeOH (40 : 1)	56	19

a) Yield from harmalane.

Fig. 1. Two Intermediates

m/*z* 344 in the MS and ^{1}H-NMR signals at δ 4.00 (3H, s, OMe), 8.03 (1H, s, 14-H), 7.45 (1H, t, *J* = 8 Hz, 18-H), 8.37 (1H, dd, *J* = 8, 1.5 Hz, 17-H), and 8.70 (1H, br d, *J* = 8 Hz, 19-H), establishing the structure of **3b** bearing an ester group at the 16-position. This lactam **3b** corresponds to the known alkaloid oxogambirtannine,[7] which had been already synthesized by several groups,[5,8] though most of the syntheses would not be suitable for large-scale practical synthesis, except Martin's synthesis.[8d] Recently, oxogambirtannine **3b** has been reported[9] to act as an immunostimulator in lung infection induced by *L. monocytogenes*. The minor product **4b** was also characterized from the spectral data [*m*/*z* 374 (M^+), δ 3.94 and 4.10 (each 3H, s, OMe, COOMe), 6.61 (1H, s, 14-H), 7.97 (1H, d, *J* = 8 Hz, 17-H), and 7.26 (1H, d, *J* = 8 Hz, 16-H)], which firmly established the 18,19-disubstituted benz[*g*]indolo[2,3-*a*]quinolizine structure. Although the ratio of the desired lactam **3b** was slightly improved by using either ether or benzene as a solvent for irradiation, it is quite exceptional to have observed that photocyclization of the *o*-methoxyenamide **2b** showed low regioselectivity in the direction of cyclization. This result can be explained as follows[6]; in the case of the enamide having an electron-attracting group such as a methoxycarbonyl group in addition to a methoxyl group at the *o*-position, the *o*-methoxyl group would contribute to the resonance with not only the amide carbonyl but also the *o*-methoxycarbonyl group, resulting in the formation of two intermediates, A and B in Fig. 1.

Thus, the photocyclization would proceed to afford a mixture of two lactams **3b** (major) and **4b** (minor) as a result of the preferential contribution of an excited from A over the other form B.

Since direct conversion of the ester **3b** to the aldehyde **3a** by partial reduction with diisobutylaluminum hydride was unsuccessful, we investigated a two-step conversion *via* the corresponding alcohol **3c**. Reduction of the ester **3b** with sodium borohydride at refluxing temperature in a mixture of methanol–tetrahydrofuran (THF) (7 : 1) for 10 h quantitatively afforded the alcohol **3c**, which gave spectral data identical with those of the authentic alcohol,[3] derived from naucleficine by Mao's group. Finally, oxidation of the alcohol **3c** with manganese dioxide in chloroform afforded the desired aldehyde **3a** in 66% yield. This was found by direct comparison to be identical with an authentic sample of the natural alkaloid,[3] naucleficine, provided by Professor Mao. Thus, we have completed the first total synthesis of naucleficine in four steps from harmalane, in a total yield of 38%.

Experimental

The ^{1}H-NMR spectra were measured with JEOL PMX-60 (60 MHz) and Varian XL-200 (200 MHz) instruments for solutions in deuteriochloroform (with tetramethylsilane as an internal reference), and the infrared (IR) spectra were measured with a Hitachi 270-30 machine for solutions in chloroform unless otherwise stated. MS were taken with a Hitachi M-80 spectrometer. All melting points were determined with a Kofler-type hot-stage apparatus and are uncorrected. Extracts from the reaction mixture were washed with water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. Photochemical reactions were carried out by irradiation with a high-pressure (100 or 300 W) mercury lamp through a Pyrex filter (Eikosha, Osaka, Japan, PIH-100 or PIH-300); during irradiation, the solutions were kept at 0—5 °C, whilst being stirred and bubbled through with nitrogen. Thin layer chromatography (TLC) was performed on pre-coated Silica gel 60F-254 plates (0.25 mm thick, Merck) and preparative TLC (p-TLC) on pre-coated Silica gel 60F-254 plates (0.5 mm thick, Merck), and spots were detected by ultraviolet (UV) irradiation of the plate at 254 and 300 nm or by exposure to iodine vapor. Short column chromatography (SCC) was undertaken on a short glass filter with Silica gel 60F-254 (Merck) under reduced pressure using an aspirator.

Methyl 3,14,15,16,17,18,19,20-Octadehydro-21-oxoyohimban-18-carboxylate (4a) A mixture of the isophthalic acid monopotassium salt (523 mg) and thionyl chloride (5.4 ml) in benzene (11 ml) was refluxed for 1.5 h. The cooled reaction mixture was filtered and the filtrate was concentrated to give an oil. Benzene was added and then evaporated to give the acid chloride **1a**. A solution of the crude acid chloride **1a** in benzene (20 ml) was added to a solution of harmalane (368 mg) and triethylamine (0.4 ml) in benzene (20 ml) with stirring at 0 °C. After being stirred at room temperature for 1 h, the reaction mixture was filtered. The filtrate was diluted with either benzene or acetonitrile (300 ml) and the resulting solution was irradiated for 1 h. The solvent was evaporated off under reduced pressure and the resulting residue was triturated with acetonitrile to give a solid, which was recrystallized from methylene dichloride to give the lactam **4a** as yellow crystals, mp > 280 °C, in 80—81% yield. IR (Nujol): 3250 (NH), 1710 (COOMe), 1650 (NCO) cm^{-1}. ^{1}H-NMR (200 MHz, dimethylsulfoxide (DMSO)-d_6) δ: 8.84 (1H, d, *J* = 1.5 Hz, 19-H), 8.20 (1H, dd, *J* = 8, 1.5 Hz, 17-H), 7.74 (1H, d, *J* = 8 Hz, 16-H), 7.15 (1H, s, 14-H), 4.45 (2H, t, *J* = 7 Hz, 5-H_2), 3.94 (3H, s, COOMe), 3.14 (2H, t, *J* = 7 Hz, 6-H_2). High-resolution MS *m*/*z*: Calcd for $C_{21}H_{16}N_2O_3$ (M^+) 344.116. Found: 344.115. *Anal.* Calcd for $C_{21}H_{16}N_2O_3 \cdot 1/5CH_2Cl_2$: C, 70.47; H, 4.58; N, 7.75. Found: C, 70.34; H, 4.49; N, 7.97.

Dimethyl 2-Methoxyisophthalate (1c) According to the literature,[10] 2-hydroxyisophthalic acid (**1b**) was prepared by oxidation of 2-hydroxy-3-methylbenzoic acid with lead dioxide. A mixture of methyl iodide (4 ml), anhydrous potassium carbonate (1 g), and the acid **1b** (355 mg) in anhydrous acetone (10 ml) was heated at 40—50 °C for 4—5 h, then allowed to cool. Potassium carbonate was filtered off and the filtrate was concentrated to give a residue, which was extracted with methylene dichloride. The extract was washed, dried, and evaporated to give a residue, which was distilled to afford the diester **1c** (163 mg, 76%) as a colorless oil, bp 160—170 °C (4—5 mmHg). IR: 1730 (COOMe) cm^{-1}. ^{1}H-NMR (60 MHz) δ: 7.83 (2H, d, *J* = 8 Hz, 4- and 6-H), 7.12 (1H, dd, *J* = 10, 8 Hz, 5-H), 3.90 (9H, s, COOMe × 2 and OMe). High-resolution MS *m*/*z*: Calcd for $C_{11}H_{12}O_5$ (M^+) 224.068. Found: 224.069.

2,3,4,9-Tetrahydro-2-(2-methoxy-3-methoxycarbonylbenzoyl)-1-methylene-1*H*-pyrido[3,4-*b*]indole (2b) A mixture of the diester **1c** (1.2 g) and potassium hydroxide (370 mg) in methanol (17.3 ml) was refluxed for 3.5 h. Evaporation of the solvent gave a residue, which was washed with hexane–ether (1 : 1) to afford the colorless potassium salt. Treatment of the salt with thionyl chloride as described for **4a** and usual work-up gave the acid chloride **1d**. A solution of the acid chloride **1d** in benzene (40 ml) was added to a solution of harmalane (720 mg) and triethylamine (580 mg) in benzene (250 ml) with stirring at 0 °C. After being stirred at room temperature for 1 h, the reaction mixture was filtered. The filtrate was

concentrated to give the unstable enamide **2b** (1.18 g, 80%) as a yellow oil, which was irradiated without purification. ^{1}H-NMR (60 MHz) δ: 5.86 and 5.01 (each 1H, br s, $C=CH_2$), 3.80 (6H, s, OMe and COOMe).

Photocyclization of the Enamide 2b According to the irradiation procedure described for **2a**, a solution of the unstable crude enamide **2b** (143 mg) was irradiated for 1.5 h in the solvents shown in Table I. Evaporation of the solvent and purification of the crude product by SCC (methanol : methylene dichloride = 3 : 97) gave oxogambirtannine (**3b**), mp 206—209 °C (lit.[8b] 201—205 °C) (yellow crystals from methanol) and methyl 3,14,15,16,17,18,19,20-octadehydro-19-methoxy-21-oxoyohimban-18-carboxylate (**4b**), mp 251—257 °C (pale yellow crystals from hexane–methanol) in the yields shown in Table I. **3b** showed IR, MS, and NMR spectra identical with those of an authentic sample.[7] **3b**: IR: 1700 (COOMe), 1640 (NCO) cm^{-1}. ^{1}H-NMR (200 MHz) δ: 8.94 (1H, br s, NH), 8.70 (1H, br d, J=8 Hz, 19-H), 8.37 (1H, dd, J=8, 1.5 Hz, 17-H), 8.03 (1H, s, 14-H), 7.45 (1H, t, J=8 Hz, 18-H), 4.55 (2H, t, J=8 Hz, 5-H_2), 4.00 (3H, s, COOMe), 3.16 (2H, t, J=8 Hz, 6-H_2). High-resolution MS *m/z*: Calcd for $C_{21}H_{16}N_2O_3$ (M^+) 344.116. Found: 344.117. *Anal.* Calcd for $C_{21}H_{16}N_2O_3$: C, 73.24; H, 4.68; N, 8.14. Found: C, 73.31; H, 4.49; N, 8.35. **4b**: IR: 1720 (COOMe), 1650 (NCO) cm^{-1}. ^{1}H-NMR (200 MHz) δ: 8.45 (1H, br s, NH), 7.97 (1H, d, J=8 Hz, 17-H), 7.26 (1H, d, J=8 Hz, 16-H), 6.61 (1H, s, 14-H), 4.52 (2H, t, J=6.5 Hz, 5-H_2), 4.10 and 3.96 (each 3H, s, COOMe and OMe), 3.16 (2H, t, J=6.5 Hz, 6-H_2). High-resolution MS *m/z*: Calcd for $C_{22}H_{18}N_2O_4$ (M^+) 374.127. Found: 374.127. *Anal.* Calcd for $C_{22}H_{18}N_2O_4 \cdot 1/2H_2O$: C, 68.92; H, 5.00; N, 7.31. Found: C, 68.99; H, 4.65; N, 7.60.

3,14,15,16,17,18,19,20-Octadehydro-21-oxoyohimban-16-methanol (3c) A solution of the ester **3b** (51 mg) and sodium borohydride (300 mg) in a mixture of methanol–THF (7 : 1) (23 ml) was refluxed for 10 h. Evaporation of the solvent gave a residue, which was dissolved in water (100 ml). The solution was acidified by the addition of 10% hydrochloric acid and cooled. The separated crystals were collected by filtration and recrystallized from water–methanol to give the alcohol (**3c**, 46.5 mg, 99%) as colorless crystals, mp 263—266 °C (lit.[3] 280—283 °C (dec.)), which showed MS and IR spectra identical with those of the authentic alcohol.[3] IR (Nujol): 3200 (NH and OH), 1640 (NCO) cm^{-1}. High-resolution MS *m/z*: Calcd for $C_{20}H_{16}N_2O_2$ (M^+) 316.121. Found: 316.120.

Naucleficine (3a) A mixture of the alcohol **3c** (30 mg) and manganese dioxide (95 mg) in a mixture of chloroform–THF (3 : 1) was refluxed with stirring for 30 min. Manganese dioxide was filtered off and the filtrate was concentrated to give a residue. Purification of the residue by p-TLC (hexane : AcOEt = 1 : 1) afforded the starting alcohol **3c** (8.3 mg) and naucleficine (**3a**) (19.6 mg, 66%), mp 284—290 °C (orange crystals from methylene dichloride–methanol), which was identical with an authentic sample[3] provided by Professor Mao, upon direct comparison of their MS, IR, and NMR spectra. IR (Nujol): 3350 (NH), 1680 (CHO), 1640 (NCO) cm^{-1}. ^{1}H-NMR (200 MHz) δ: 12.80 (1H, s, CHO), 8.86 (1H, br s, NH), 8.75 (1H, br d, J=8 Hz, 19-H), 8.22 (1H, s, 14-H), 8.10 (1H, dd, J=8, 1.5 Hz, 17-H), 4.58 (2H, t, J=7 Hz, 5-H_2), 3.19 (2H, t, J=7 Hz, 6-H_2). High-resolution MS *m/z*: Calcd for $C_{20}H_{14}N_2O_2$ (M^+) 314.106. Found: 314.106.

Acknowledgements We thank Professor L. Mao of the Chinese Academy of Medical Sciences, People's Republic of China, for his generous gift of the authentic sample of naucleficine. We are grateful to Misses Y. Fujioka and N. Uda for their technical assistance.

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Synthesis of (3*S*,4*S*)-3,4-Dihydroxyprolines from L-Tartaric Acid

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Natural (2*S*,3*S*,4*S*)-3,4-dihydroxyproline (1) and the new (2*R*,3*S*,4*S*)-isomer (7) have been synthesized from L-tartaric acid *via* cyanosilylation of the cyclic Schiff base.

Keywords (2*S*,3*S*,4*S*)-3,4-dihydroxyproline; (2*R*,3*S*,4*S*)-3,4-dihydroxyproline; L-tartaric acid; (3*S*,4*S*)-3,4-dihydroxypyrrolidine; NOE difference spectrum; *N*-chlorosuccinimide; 1,8-diazabicyclo[5.4.0]-7-undecene; cyclic Schiff base; cyanotrimethylsilane; zinc iodide; diastereoselectivity

Eight stereoisomers can exist in 3,4-dihydroxyproline systems due to the presence of three chiral carbons. Two isomers have been isolated from nature, (2*S*,3*S*,4*S*)-**1** from diatom cell walls[1] and (2*S*,3*R*,4*R*)-**2** from *Amanita virosa* mushrooms,[2] and they have been synthesized from 3,4-dehydroproline,[3] from diacetone glucose,[4] and from 2-amino-3-hydroxy-4-pentenoic acid.[5] Recently, four unnatural isomers have been synthesized by Fleet and coworkers, (2*R*,3*S*,4*R*)-**3** from D-ribonolactone,[6] (2*R*,3*R*,4*R*)-**4** from D-glucuronolactone,[7] (2*S*,3*S*,4*R*)-**5** from D-glucose,[8] and (2*S*,3*R*,4*S*)-**6** from D-gulonolactone.[9] The remaining two isomers (2*R*,3*S*,4*S*)-**7** and (2*R*,3*R*,4*S*)-**8** have not yet been synthesized.

Since 3,4-dihydroxyprolines are relatively new compounds, few studies have yet been done on their biological activities, but it is well known that polyhydroxylated pyrrolidine systems have a number of biological activities,[10] *e.g.* inhibition of glycosidases[11] as well as inhibition of human immunodeficiency virus (HIV) replication.[12] In fact, (2*S*,3*R*,4*R*)-**2**, which is an enantiomer of the unknown compound **7**, has been reported to be a potent and specific β-D-glucuronidase inhibitor.[13]

We wish to describe here the synthesis of (3*S*,4*S*)-3,4-dihydroxyprolines (**1** and **7**) from the cheap chiral starting material, L-tartaric acid (Chart 1). Our synthetic plan includes syntheses of pyrrolidine derivatives, oxidation of the amine to form the cyclic Schiff bases, and cyanosilylation (Strecker synthesis).

L-Tartaric acid was easily converted into (3*S*,4*S*)-1-benzyl-3,4-dihydroxypyrrolidine (**10**) in 42% yield *via* (3*R*,4*R*)-1-benzyl-3,4-dihydroxy-2,5-dioxopyrrolidine (**9**) by a slight modification of the reported method.[14] In order to protect the hydroxy groups of **10**, it was converted into the benzoyl (Bz) ester **11a** and the *tert*-butyldimethylsilyl (TBDMS) ether **11b** in 83 and 92% yields respectively. Hydrogenolysis of **11a** (20% $Pd(OH)_2/C$, AcOH, 4 atm, 24 h) quantitatively yielded (3*S*,4*S*)-3,4-dibenzoyloxypyrrolidine (**12a**), which was stored as the acetate form because of the instability of the amino ester. Hydrogenolysis of **11b** (20% $Pd(OH)_2/C$, MeOH, 4 atm, 6 h) was somewhat complicated and purification of the product by column chromatography on silica gel was necessary to give (3*S*,4*S*)-3,4-bis(*tert*-butyldimethylsilyloxy)pyrrolidine (**12b**) in 83% yield. Treatment of **12a** and **12b** with *N*-chlorosuccinimide (NCS) in Et_2O caused *N*-chlorination to give **13a** and **13b** in 91 and 92% yields. Then **13a** and **13b** were dehydrochlorinated with 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) in benzene to give the cyclic Schiff bases **14a** and **14b**, which were not isolated, since it is well known that non-conjugated Schiff bases easily polymerize.[15] The benzene solutions of **14a** and **14b** were well washed with water to remove DBU,[16] dried and concentrated to *ca.* 4% solutions. Reaction of **14a** with 3 eq of cyanotrimethylsilane in benzene in the presence of 6 mol% of zinc iodide at room temperature for 24 h followed by treatment with water provided an epimeric mixture of aminonitriles **15a** in 85% yield. In the case of using zinc chloride as a Lewis acid in place of zinc iodide, no formation of **15a** was observed. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy showed that **15a** consists of *ca.* 40% of 2,3-*cis* and *ca.* 60% of 2,3-*trans* isomers. In general, it is difficult to determine the stereochemistry of substituted pyrrolidine systems by using the chemical shifts and coupling constants when their conformations are flexible.[17] But the ^{1}H–^{1}H nuclear Overhauser effect (NOE) difference spectra of the compounds (**10**, **11a**, **b**, **12b**) showed that irradiation of their 3,4-protons caused greater increase of the magnitude of the signals of 2,5-protons at low fields than at high fields

HO OH / N H COOH — 1, 2, 3, 4, 5, 6, 7, 8

Fig. 1

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reagents: a) $PhCH_2NH_2$, xylene; b) $LiAlH_4$, THF; c) 11a–BzCl, aq. Na_2CO_3, CH_2Cl_2; 11b–NaH, TBDMSCl; d) H_2, $Pd(OH)_2/C$; e) NCS, Et_2O; f) DBU, PhH; g) Me_3SiCN, ZnI_2; h) Ac_2O; i) 6 N HCl, AcOH; j) MeOH, $SOCl_2$; k) CbzCl, dioxane, aq. $NaHCO_3$; l) 1 N NaOH, MeOH; m) Amberlite 200C; n) H_2, Pd/C

Chart 1

(Fig. 2). These results suggest that the pyrrolidine systems in this paper should obey the rule for the cyclopropane systems[18]; the substituents generally tend to cause signals of the protons *cis* to them to appear at higher fields than those *trans* to them, and the vicinal coupling constants between *cis* protons are larger than those between *trans*

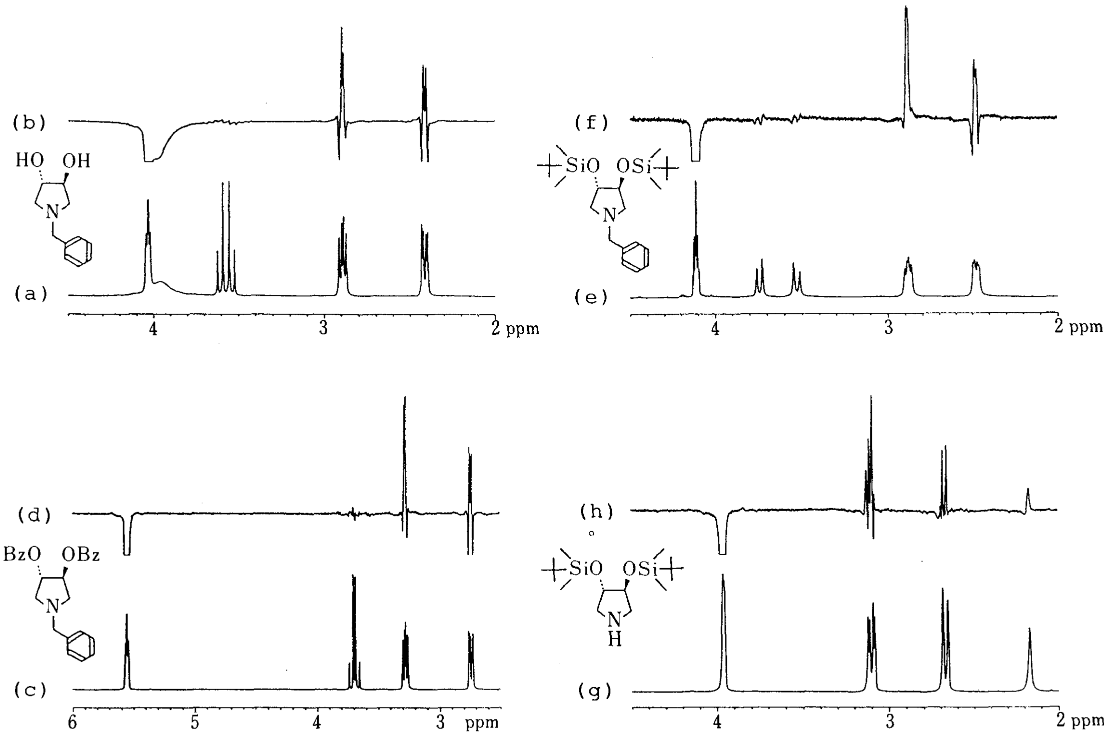

Fig. 2. NOE Difference Spectra of **10**, **11a**, **11b**, and **12b** in $CDCl_3$

(a), (c), (e), (g) Off-resonance gated irradiation spectra (control) of **10**, **11a**, **11b**, and **12b**, respectively; (b), (d), (f), (h) NOE difference spectra between on- and off-resonance.

protons, so the signal at δ 4.70 (0.4H, d, $J=6$ Hz) was assigned to the proton at the 2-position of 2,3-*cis*-**15a**, and the signal at δ 4.26 (0.6H, d, $J=1$ Hz) was assigned to that of 2,3-*trans*-**15a**.

In order to confirm the ratio of the diastereoisomers and to separate them from each other, the aminonitrile **15a** was treated with acetic anhydride to give an epimeric mixture of *N*-acetyl derivatives, which was separated by preparative silica gel thin layer chromatography (PLC) to yield 36% of 2,3-*cis* (**16**) and 60% of 2,3-*trans* (**17**) isomers. Although the addition reactions of cyanotrimethylsilane to acyclic Schiff bases have high diastereoselectivities,[19] in the case of **14a**, the diastereoselectivity was lower. Separation of the epimers as *N*-acetyl forms seemed to be difficult, for these isomers have similar *Rf* values on silica gel thin layer chromatography (TLC). So we decided to hydrolyze **15a** without separation of the epimers. Benzene solution of **14b** was treated in the same way as described for **14a** to give an epimeric mixture of aminonitriles **15b** in 90% yield, and ^{1}H-NMR analysis showed the product to consist of *ca.* 40% of 2,3-*cis* and *ca.* 60% of 2,3-*trans* isomers. Judging from these results, the low stereoselectivities of the addition reactions seem to be attributable to the substituents at the 4-positions, which prevent the formations of 2,3-*trans* isomers.

Compound **15a** was heated in AcOH–6 N HCl at 100 °C for 24 h and esterification followed by *N*-benzyloxycarbonylation of the resulting epimeric mixture of amino acids gave a mixture of epimers **18** and **19**. The mixture was easily separated on silica gel column chromatography to give **18** in 17% yield and **19** in 26% yield. The yields were not high because the benzoyl groups at the 3,4-positions could not be completely removed at the hydrolysis stage. Compound **15b** was heated in AcOH–6 N HCl at 100 °C for 6 h and was worked up in the same way as **15a** to give **18** in 28% yield and **19** in 42% yield. Compound **18** was deprotected, hydrolyzed with 1 N KOH, ion-exchanged, and hydrogenolyzed to give (2*S*,3*S*,4*S*)-3,4-dihydroxyproline (**1**) quantitatively; the physical data for the product, including the specific rotation, were identical with those reported for natural **1**. Compound **19** was also quantitatively deprotected in the same way as **18** to give the new compound (2*R*,3*S*,4*S*)-3,4-dihydroxyproline (**7**), of which physical data were almost identical with those reported for the enantiomer **2** except for the sign of the specific rotation.

In conclusion, the natural product (2*S*,3*S*,4*S*)-dihydroxyproline (**1**) and the new compound (2*R*,3*S*,4*S*)-3,4-dihydroxyproline (**7**) were synthesized from L-tartaric acid.

Experimental

Melting points were measured with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 polarimeter. NMR spectra were recorded in chloroform-*d* ($CDCl_3$) solution, except for amino acids **1** and **7**, on a JEOL PMX-60SI, a FX-100 or a GSX-400 spectrometer with tetramethylsilane as an internal standard. As for **1** and **7**, they were analyzed in deuterium oxide (D_2O) with dioxane as an internal standard (δ 3.7 for ^{1}H-NMR and δ 67.4 for ^{13}C-NMR). The ^{1}H–^{1}H NOE experiments in the difference mode were performed at 25 °C for 30—60 mg of sample in 0.6 ml of $CDCl_3$. Infrared (IR) spectra were recorded on a Hitachi 270-30 spectrophotometer. Mass spectra (MS) were obtained with a JEOL JMS-DX300 instrument. TLC was performed on Silica gel 60 F_{254} plates (0.25 mm, Merck) or Aluminium oxide 60 F_{254} plates (0.25 mm, Merck). PLC was performed on Silica gel 60 plates (2 mm, Merck, Art. 5717). Column chromatography was performed on silica gel (Kieselgel 60, 70—230 mesh, Merck) or alumina (Aluminium oxide 90, 70—230 mesh, Merck). Flash chromatography was performed on silica gel (Silica gel 60, 230—400 mesh, Nacalai Tesque).

(3*R*,4*R*)-1-Benzyl-3,4-dihydroxy-2,5-dioxopyrrolidine (9) This compound was synthesized according to Wong's method[14a] with some modification. In order to prevent the formation of diamide, an equimolar amount of benzylamine with respect to L-tartaric acid was employed, and after recrystallization of the product from water, recrystallization from EtOH gave **9** in 60% yield. Colorless needles, mp 197—199 °C (lit.,[14a] mp 196—198 °C), $[\alpha]_D^{22}$ +138.0° (*c*=1.0, MeOH) [lit.,[14a] $[\alpha]_D^{25}$ +126° (MeOH)].

(3*S*,4*S*)-1-Benzyl-3,4-dihydroxypyrrolidine (10) The report on this compound[14b] includes no experimental details. The imide **9** (11.0 g, 50 mmol) was slowly added to a suspension of $LiAlH_4$ (5.1 g, 134 mmol) in tetrahydrofuran (THF) (300 ml) with stirring at 0 °C under nitrogen and the mixture was refluxed for 12 h, then cooled to room temperature. AcOEt (12 ml) was added dropwise to the mixture and cooled in an ice bath. Then water (5.1 ml), 15% NaOH (5.1 ml) and water (15.3 ml) were successively added dropwise to the reaction mixture with vigorous stirring. The precipitate was filtered off with Hyflo super-cel (Johns-Manville) and well washed with hot THF (100 ml × 2). The filtrate was concentrated under reduced pressure and the residue was separated by column chromatography on alumina (300 g, $CHCl_3$–MeOH, 20:1) to give 6.7 g (70%) of **10** as a pale yellow oil, which soon solidified. Recrystallization from AcOEt gave colorless prisms, mp 109—110 °C, $[\alpha]_D^{22}$ +8.3° (*c*=1.1, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 2.42 (2H, dd, *J*=9.9, 4.0 Hz, *trans*-2-H and *trans*-5-H), 2.90 (2H, dd, *J*=9.9, 5.8 Hz, *cis*-2-H and *cis*-5-H), 3.55 and 3.60 (2H, each d, *J*=12.8 Hz, $PhCH_2$), 3.94 (2H, br s, 2×OH), 4.03 (2H, m, 2×O–CH=), 7.26 (5H, m, aromatic H). ^{13}C-NMR (100 MHz) δ: 60.18 (t), 78.39 (d), 127.36 (d), 128.34 (d), 129.20 (d), 137.49 (s). IR ν_{max}^{neat} cm^{-1}: 3364 (OH). MS *m/z*: 193 (M^+). *Anal.* Calcd for $C_{11}H_{15}NO_2$: C, 68.37; H, 7.82; N, 7.25. Found: C, 68.40; H, 8.00; N, 7.26.

(3*S*,4*S*)-3,4-Dibenzoyloxy-1-benzylpyrrolidine (11a) Benzoyl chloride (20 ml) was added dropwise to a mixture of a solution of **10** (13.5 g, 70 mmol) in CH_2Cl_2 (100 ml) and a solution of Na_2CO_3 (18.1 g, 170 mmol) in water (100 ml) with vigorous stirring at 0 °C and the mixture was stirred at room temperature for 2 h. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (100 ml × 2). The combined CH_2Cl_2 solution was washed with water (100 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting oil was separated by column chromatography on alumina (300 g, $CHCl_3$) to give 27.0 g of **11a** (96%) as a white powder, mp 108—110 °C, which was recrystallized from diisopropyl ether. Colorless needles, mp 110—112 °C, $[\alpha]_D^{22}$ +86.6° (*c*=0.51, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 2.74 (2H, dd, *J*=10.3, 4.4 Hz, *trans*-2-H and *trans*-5-H), 3.27 (2H, dd, *J*=10.3, 6.2 Hz, *cis*-2-H and *cis*-5-H), 3.68 and 3.70 (2H, each d, *J*=13.2 Hz, $PhCH_2$), 5.55 (2H, m, 2×O–CH=), 7.21—7.60 (11H, m, aromatic H), 8.00—8.10 (4H, m, aromatic H). ^{13}C-NMR (25 MHz) δ: 58.36 (t), 59.83 (t), 78.48 (d), 127.42 (d), 128.54 (d), 128.95 (d), 129.95 (d), 133.36 (d), 137.95 (s), 166.24 (s). IR ν_{max}^{KBr} cm^{-1}: 1726 (CO), 1278, 1112. MS *m/z*: 402 (MH^+), 401 (M^+). *Anal.* Calcd for $C_{25}H_{23}NO_4$: C, 74.80; H, 5.77; N, 3.49. Found: C, 74.70; H, 5.81; N, 3.47.

(3*S*,4*S*)-1-Benzyl-3,4-bis(*tert*-butyldimethylsilyloxy)pyrrolidine (11b) Compound **10** (2.5 g, 13 mmol) was slowly added to a suspension of 60% NaH (2.0 g, 50 mmol) in THF (100 ml) under nitrogen at room temperature. After the mixture turned yellow, a solution of *tert*-butyldimethylchlorosilane (6.0 g, 40 mmol) in THF (50 ml) was added dropwise to it and the mixture was stirred at room temperature for 1 h, refluxed for 1 h, then cooled in an ice bath. Water (100 ml) was slowly added and THF was evaporated off under reduced pressure. The residual solution was extracted with benzene (100 ml × 2). The benzene layer was washed with water (100 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to column chromatography on alumina (300 g, hexane, then benzene) to give 5.0 g of **11b** (92%) as a colorless oil, $[\alpha]_D^{25}$ +58.3° (*c*=1.0, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 0.01—0.10 (12H, m, 4×Si–CH_3), 0.88 (18H, s, 2×$C(CH_3)_3$), 2.45 (2H, dd, *J*=9.5, 4.8 Hz, *trans*-2-H and *trans*-5-H), 2.86 (2H, dd, *J*=9.5, 6.2 Hz, *cis*-2-H and *cis*-5-H), 3.51 and 3.71 (2H, each d, *J*=13.2 Hz, $PhCH_2$), 4.11 (2H, m, 2×O–CH=), 7.30 (5H, s, aromatic H). ^{13}C-NMR (100 MHz) δ: −4.70 (q), −4.60 (q), 18.02 (s), 25.86 (q), 60.72 (t), 60.78 (t), 79.82 (d), 126.86 (d), 128.18 (d), 128.67 (d), 138.73 (s). IR ν_{max}^{neat} cm^{-1}: 1102 (C–O–Si). MS *m/z*: 421 (M^+).

(3*S*,4*S*)-3,4-Dibenzoyloxypyrrolidine (12a) Compound **11a** (10.0 g, 25 mmol) was hydrogenolyzed in AcOH (100 ml) in the presence of 20% $Pd(OH)_2$/C (1.0 g) at 4 atm for 24 h. The catalyst was filtered off and washed with AcOH, and the filtrate was concentrated under reduced pressure to give 9.25 g of the acetic acid salt of **12a** (100%) as a white powder. The obtained salt (2.00 g, 5.4 mmol) was dissolved in MeOH (10 ml) and 1 N KOH (5.4 ml) was added slowly with stirring, followed by addition of water (100 ml). The whole was extracted with benzene (100 ml × 2), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give 1.67 g of **12a** (100%) as a pale yellow oil. ^{1}H-NMR (60 MHz) δ: 2.03 (1H, s, NH), 3.17 (2H, dd, *J*=12.5, 2.5 Hz, *trans*-2-H and *trans*-5-H), 3.56 (2H, dd, *J*=12.5, 5 Hz, *cis*-2-H and *cis*-5-H), 5.53 (2H, m, 2×O–CH=), 7.23—7.69 (6H, m, aromatic H), 8.00—8.23 (4H, m, aromatic H). ^{13}C-NMR (100 MHz) δ: 52.56 (t), 79.38 (d), 128.34 (d), 128.45 (d), 129.64 (d), 129.72 (s), 133.33 (d), 165.71 (s). IR ν_{max}^{neat} cm^{-1}: 3352 (NH), 1722 (CO), 1276, 1112. For further analysis, compound **12a** was treated with di-*tert*-butyl dicarbonate (Boc_2O) in $CHCl_3$ to give (3*S*,4*S*)-3,4-dibenzoyloxy-1-*tert*-butoxycarbonylpyrrolidine quantitatively. Colorless needles (EtOH), mp 178.5—179 °C, $[\alpha]_D^{25}$ +56.1° (*c*=0.55, $CHCl_3$). ^{1}H-NMR (60 MHz) δ: 1.48 (9H, s, 3×CH_3), 3.50—4.23 (4H, m, 2×CH_2), 5.61 (2H, m, 2×O–CH=), 7.30—7.77 (6H, m, aromatic H), 8.00—8.37 (4H, m, aromatic H). IR ν_{max}^{KBr} cm^{-1}: 1732 (CO), 1688 (CO), 1262, 1106. MS *m/z*: 412 (MH^+). *Anal.* Calcd for $C_{23}H_{25}NO_6$: C, 67.14; H, 6.12; N, 3.40. Found: C, 67.29; H, 6.20; N, 3.44. For another analysis, compound **12a** was led to the picrate. Yellow needles (MeOH), mp 233—235 °C. *Anal.* Calcd for $C_{24}H_{20}N_4O_{11}$: C, 53.34; H, 3.73; N, 10.37. Found: C, 53.37; H, 3.56; N, 10.31.

(3*S*,4*S*)-3,4-Bis(*tert*-butyldimethylsilyloxy)pyrrolidine (12b) Compound **11b** (3.2 g, 7.8 mmol) was hydrogenolyzed in MeOH (18 ml) in the presence of 20% $Pd(OH)_2$/C (0.35 g) at 4 atm for 6 h. The catalyst was filtered off and washed with EtOH, then the filtrate was concentrated under reduced pressure to give a dark brown oil, which was subjected to column chromatography on silica gel (140 g, Et_2O, then Et_2O–Et_3N, 49:1) to give 2.1 g of **12b** (83%) as a pale yellow oil, $[\alpha]_D^{25}$ +35.9° (*c*=0.88, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 0.01—0.20 (12H, m, 4×Si–CH_3), 0.87 (18H, s, 2×$C(CH_3)_3$), 2.14 (1H, s, NH), 2.67 (2H, dd, *J*=12.5, 1.5 Hz, *trans*-2-H and *trans*-5-H), 3.10 (2H, dd, *J*=12.5, 4.0 Hz, *cis*-2-H and *cis*-5-H), 3.96 (2H, m, 2×O–CH=). ^{13}C-NMR (100 MHz) δ: −4.68 (q), 17.97 (s), 25.79 (q), 54.22 (t), 79.38 (d). IR ν_{max}^{neat} cm^{-1}: 1110 (C–O–Si). MS *m/z*: 331 (M^+).

(3*S*,4*S*)-3,4-Dibenzoyloxy-1-chloropyrrolidine (13a) NCS (0.72 g, 5.4 mmol) was added to a solution of **12a** (1.67 g, 5.4 mmol) in Et_2O (20 ml) under argon at room temperature and the mixture was stirred for 2 h. The whole was extracted with Et_2O (40 ml) and water (40 ml). The aqueous layer was extracted with Et_2O (40 ml × 2) and the combined Et_2O layer was washed with 10% $Na_2S_2O_3$ (40 ml), then water (40 ml), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel (20 g, benzene) to give 1.70 g of **13a** (91%) as a colorless oil. ^{1}H-NMR (60 MHz) δ: 3.45 (2H, dd, *J*=12, 4 Hz, *trans*-2-H and *trans*-5-H), 3.86 (2H, dd, *J*=12, 6 Hz, *cis*-2-H and *cis*-5-H), 5.68 (2H, m, 2×O–CH=), 7.33—7.83 (6H, m, aromatic H), 8.00—8.33 (4H, m, aromatic H). ^{13}C-NMR (25 MHz) δ: 66.53 (t), 77.65 (d), 128.65 (d), 129.36 (d), 130.01 (d), 133.71 (s), 165.95 (s). IR ν_{max}^{neat} cm^{-1}: 1728 (CO), 1274, 1112. MS *m/z*: 345 (M^+).

(3*S*,4*S*)-3,4-Bis(*tert*-butyldimethylsilyloxy)-1-chloropyrrolidine (13b) This compound was obtained as a colorless oil from **12b** in 92% yield in the same way as **13a**. ^{1}H-NMR (60 MHz) δ: 0.03 (12H, s, 4×Si–CH_3), 0.86 (18H, s, 2×$C(CH_3)_3$), 3.07 (2H, dd, *J*=10, 4.8 Hz, *trans*-2-H and *trans*-5-H), 3.50 (2H, dd, *J*=10, 5.6 Hz, *cis*-2-H and *cis*-5-H), 4.10 (2H, m, 2×O–CH=). ^{13}C-NMR (100 MHz) δ: −4.59 (q), −4.45 (q), 18.17 (s), 25.97 (q), 69.37 (t), 79.25 (d). IR ν_{max}^{neat} cm^{-1}: 1130 (C–O–Si). MS *m/z*: 350 ($M^+ - CH_3$), 329 (M^+ −HCl).

An Epimeric Mixture of (3*S*,4*S*)-3,4-Benzoyloxy-2-cyanopyrrolidines (15a) A solution of **13a** (1.70 g, 4.9 mmol) in benzene (30 ml) was added to a solution of DBU (1.0 g, 6.6 mmol) in benzene (20 ml) and stirred overnight at room temperature. After addition of benzene (70 ml), the whole was washed with water (100 ml × 3). The aqueous layer was extracted with benzene (100 ml). The combined benzene layer was dried over anhydrous Na_2SO_4 and concentrated to 40 ml under reduced pressure at 30 °C. Cyanotrimethylsilane (2 ml, 16 mmol) and zinc iodide (100 mg, 0.3 mmol) were added to the solution and the mixture was stirred at room temperature for 24 h. Dioxane (20 ml) and water (20 ml) were then added and the reaction mixture was stirred for 1 h. After addition of water (20 ml), the whole was extracted with benzene (40 ml × 2). The benzene layer was washed with water (100 ml), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was subjected to flash chromatography (100 g, Et_2O–hexane, 1:1) to give 1.40 g of **15a** (85%) as a colorless oil. ^{1}H-NMR (60 MHz) δ: 2.44 (1H, br s, NH), 3.10—4.00 (2H, m, N–CH_2–), 4.26 (0.6H, d, *J*=1 Hz, 2-H of *trans* isomer), 4.70 (0.4H, d, *J*=6 Hz, 2-H of *cis* isomer), 5.50—5.83 (2H, m, 2×O–CH=),

7.30—7.80 (6H, m, aromatic H), 8.00—8.33 (4H, m, aromatic H). IR ν_{max}^{neat} cm^{-1}: 2248 (CN), 1724 (CO), 1268, 1112.

An Epimeric Mixture of (3*S*,4*S*)-3,4-Bis(*tert*-butyldimethylsilyl)-2-cyanopyrrolidines (15b) Compound **13b** (1.80 g, 4.9 mmol) was treated in the same manner as **13a** but using twice the quantity of DBU. Separation by column chromatography on silica gel (100 g, Et_2O–hexane, 2:1) gave 1.58 g of **15b** (90%) as a colorless oil. ^{1}H-NMR (400 MHz) δ: 0.00—0.25 (12H, m, 4×Si–CH_3), 0.70—1.00 (18H, m, 2×$C(CH_3)_3$, 2.18 (1H, br s, NH), 2.77 (0.4H, dd, J=11.7, 1.5 Hz, *trans*-5-H of *cis* isomer), 2.95 (0.6H, dd, J=11.4, 2.2 Hz, *trans*-5-H of *trans* isomer), 3.14 (0.6H, dd, J=11.4, 4.0 Hz, *cis*-5-H of *trans* isomer), 3.31 (0.4H, dd, J=11.7, 4.4 Hz, *cis*-5-H of *cis* isomer), 3.67 (0.6H, d, J=1.8 Hz, 2-H of *trans* isomer), 4.00—4.15 (1.8H, m, 2,3,4-H of *cis* isomer and 4-H of *trans* isomer), 4.23 (0.6H, s, 3-H of *trans* isomer). IR ν_{max}^{neat} cm^{-1}: 2248 (CN), 1120 (C–O–Si). MS *m/z*: 357 (MH$^+$).

(2*R*,3*S*,4*S*)-1-Acetyl-3,4-dibenzoyloxy-2-cyanopyrrolidine (16) and (2*S*,3*S*,4*S*)-1-Acetyl-3,4-dibenzoyloxy-2-cyanopyrrolidine (17) Compound **15a** (0.67 g, 2 mmol) was heated with acetic anhydride (3 ml) at 50 °C for 0.5 h and the mixture was concentrated under reduced pressure. The residue was dissolved in benzene (50 ml), washed with saturated $NaHCO_3$ (40 ml×2) and water (50 ml), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a mixture of **16** and **17** (0.75 g) as a colorless oil in 99% yield. The epimeric mixture (100 mg) was separated by PLC (2 plates, developed 8 times with $CHCl_3$ then eluted with AcOEt) to give **16** (36 mg, lower component) and **17** (60 mg, upper component). Each of them has the same *Rf* value (0.33, $CHCl_3$–EtOH, 49:1). **16**: Colorless oil, $[\alpha]_D^{22}$ +34.3° (c=0.61, $CHCl_3$). ^{1}H-NMR (60 MHz) δ: 2.00—2.50 (3H, m, CH_3), 3.80 (1H, dd, J=11, 3.4 Hz, *trans*-5-H), 4.27 (1H, dd, J=11, 5 Hz, *cis*-5-H), 5.35 (1H, d, J=5.8 Hz, –CH(CN)–), 5.78 (2H, m, 2×O–CH=), 7.30—7.83 (6H, m, aromatic H), 8.00—8.33 (4H, m, aromatic H). IR ν_{max}^{neat} cm^{-1}: 2248 (CN), 1734 (CO), 1674 (CO), 1262, 1110. MS *m/z*: 379 (MH$^+$). **17**: Colorless oil, $[\alpha]_D^{22}$ +90.1° (c=0.89, $CHCl_3$). ^{1}H-NMR (60 MHz) δ: 2.00—2.40 (3H, m, CH_3), 3.78—4.43 (2H, m, N–CH_2–), 4.88 and 5.07 (1H, each s, –CH(CN)–), 5.79 (2H, s, 2×O–CH=), 7.30—7.80 (6H, m, aromatic H), 7.95—8.33 (4H, m, aromatic H). IR ν_{max}^{neat} cm^{-1}: 2248 (CN), 1732 (CO), 1670 (CO), 1254, 1110. MS *m/z*: 379 (MH$^+$).

(2*S*,3*S*,4*S*)-1-Benzyloxycarbonyl-3,4-dihydroxy-2-methoxycarbonylpyrrolidine (18) and (2*R*,3*S*,4*S*)-1-Benzyloxycarbonyl-3,4-dihydroxy-2-methoxycarbonylpyrrolidine (19) from 15a The aminonitrile **15a** (1.20 g, 3.6 mmol) was heated in 6 N HCl (30 ml) and AcOH (30 ml) at 100 °C for 24 h. The solution was concentrated under reduced pressure and the residue was extracted with water (100 ml) and benzene (100 ml). The aqueous layer was treated with Amberlite 200C (H$^+$ form, 150 ml), which was washed with water (500 ml) and eluted with cold 1 N NH_4OH (500 ml) and water (500 ml), then the eluate was concentrated to dryness. Thionyl chloride (20 ml) was added dropwise to MeOH (100 ml) with cooling in an ice-salt bath and stirred for 10 min. The resulting solution was added to the mixture of amino acids obtained above and stirred at room temperature for 24 h. The solution was concentrated under reduced pressure several times with additions of MeOH, then a solution of $NaHCO_3$ (2 g) in water (100 ml), dioxane (100 ml) and carbobenzyloxy chloride (CbzCl, 1 ml) were added to the residue with vigorous stirring and the whole was stirred overnight at room temperature. After evaporation of dioxane from the reaction mixture under reduced pressure, the aqueous solution was extracted with AcOEt (100 ml×2). The organic layer was washed with water (100 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was subjected to flash chromatography (200 g, $CHCl_3$–MeOH, 40:1) to give 270 mg of **19** (26%) and 180 mg of **18** (17%). **18**: Colorless oil, *Rf*=0.34 (silica gel, $CHCl_3$–MeOH, 10:1), $[\alpha]_D^{25}$ −21.4° (c=0.8, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 3.33—3.45 (1H, m, *trans*-5-H), 3.53 and 3.67 (3H, each s, CH_3, splitting due to the rotamers), 3.75—3.82 (1H, m, *cis*-5-H), 4.14—4.30 (2H, m, 3-H and 4-H), 4.30—4.50 (2H, m, 2×OH), 4.51 (1H, d, J=5.6 Hz, 2-H), 4.90—5.16 (2H, m, $PhCH_2$), 7.21—7.32 (5H, m, aromatic H). ^{13}C-NMR (100 MHz) δ: 50.95 (t), 51.40 (t), 52.25 (q), 52.48 (q), 62.69 (d), 62.74 (d), 67.52 (t), 67.56 (t), 73.78 (d), 74.29 (d), 75.97 (d), 76.65 (d), 127.69 (d), 127.81 (d), 128.15 (d), 128.45 (d), 128.51 (d), 135.90 (s), 136.01 (s), 155.13 (s), 155.47 (s), 170.65 (s), 170.74 (s). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3464 (OH), 1744 (CO), 1704 (CO), 1180, 1120, 1080. MS *m/z*: 295 (M$^+$). **19**: Colorless oil, *Rf*=0.38 (silica gel, $CHCl_3$–MeOH, 10:1), $[\alpha]_D^{25}$ +15.3° (c=1.3, $CHCl_3$). ^{1}H-NMR (400 MHz) δ: 3.46—3.52 (1H, m, *trans*-5-H), 3.54 and 3.68 (3H, each s, CH_3, splitting due to the rotamers), 3.72—3.82 (1H, m, *cis*-5-H), 3.82—4.05 (1H, m, OH), 4.07 (1H, br s, 4-H), 4.26 and 4.31 (2H, each s, 2-H and 3-H, splitting due to the rotamers), 4.30—4.45 (1H, m, OH), 4.95—5.17 (2H, m, $PhCH_2$), 7.21—7.32 (5H, aromatic H). ^{13}C-NMR (100 MHz) δ: 52.24 (t), 52.37 (t), 52.56 (q), 52.79 (q), 65.59 (d), 65.99 (d), 67.55 (t), 74.30 (d), 75.05 (d), 78.68 (d), 79.51 (d), 127.78 (d), 127.84 (d), 128.13 (d), 128.18 (d), 128.32 (d), 128.45 (d), 128.53 (d), 128.66 (d), 135.98 (s), 136.04 (s), 155.09 (s), 155.57 (s), 171.65 (s), 171.76 (s). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3460 (OH), 1706 (br, 2×CO), 1180, 1120, 1074. MS *m/z*: 295 (M$^+$).

18 and 19 from 15b The aminonitrile **15b** (1.30 g, 3.6 mmol) was heated in 6 N HCl (30 ml) and AcOH (30 ml) at 100 °C for 6 h. The reaction mixture was treated in almost the same manner as **15a** except for the omission of treatment of the crude amino acids with ion-exchange resin and the employment of one and a half times the quantity of the reagents for protection to give 452 mg of **19** (42%) and 303 mg of **18** (28%).

(2*S*,3*S*,4*S*)-3,4-Dihydroxyproline (1) A solution of **18** (180 mg, 0.61 mmol) in MeOH (4 ml) was treated with 1 N KOH (0.7 ml) at 0 °C with stirring, then the mixture was allowed to stand at room temperature for 24 h. After concentration of the solution, the residue was extracted with water (200 ml) and Et_2O (20 ml). The Et_2O layer was extracted with water (20 ml). The combined aqueous layer was washed with Et_2O (20 ml), and treated with Amberlite 200C (H$^+$ form, 20 ml), which was washed with water (40 ml) and eluted with 50% AcOH (40 ml) and water (40 ml). The eluate was concentrated under reduced pressure and the residue was hydrogenolyzed in 50% AcOH (20 ml) in the presence of 10% Pd/C (20 mg) under a pressure of 1 atm. The catalyst was filtered off and rinsed with water, then active carbon (1.5 g) was added to the combined filtrate and washing, which was again filtered. The filtrate was concentrated under reduced pressure to give **1** (90 mg) quantitatively as a white powder, mp 261 °C (dec.). Recrystallization from 90% EtOH gave colorless needles, mp 263 °C (dec.). [lit.,[1,3] mp 262 °C (dec.)], $[\alpha]_D^{27}$ −63.2° (c=0.5, H_2O) (lit.,[1] $[\alpha]_D^{20}$ −61.2°, lit.,[5] $[\alpha]_D^{22}$ −63.0°). ^{1}H-NMR (400 MHz in D_2O) δ: 3.30 (1H, d, J=12.8 Hz, *trans*-5-H), 3.66 (1H, dd, J=12.8, 3.7 Hz, *cis*-5-H), 4.34 (1H, d, J=4.0 Hz, 2-H), 4.37 (1H, d, J=3.7 Hz, 4-H), 4.42 (1H, d, J=4.0 Hz, 3-H). ^{13}C-NMR (100 MHz) δ: 51.84 (t), 66.10 (d), 75.73 (d), 76.16 (d), 171.43 (s). IR ν_{max}^{Nujol} cm^{-1}: 3368, 1634, 1570. MS *m/z*: 148 (MH$^+$). *Anal.* Calcd for $C_5H_9NO_4$: C, 40.82; H, 6.17; N, 9.52. Found: C, 40.83; H, 6.23; N, 9.60. Ninhydrin reaction: yellow.

(2*R*,3*S*,4*S*)-3,4-Dihydroxyproline (7) A solution of **19** (270 mg, 0.91 mmol) in MeOH (6 ml) was treated with 1 N KOH (1 ml) at 0 °C with stirring, then the mixture was allowed to stand at room temperature for 12 h. The reaction mixture was worked up in the same way as described for **18** using one and a half times greater amounts of the reagents to give **7** (134 mg) quantitatively as a white powder, mp 241 °C (dec.). Recrystallization from 75% EtOH gave colorless plates, mp 242 °C (dec.) [lit.,[3] mp 250 °C (dec.), data for enantiomer of **7**], $[\alpha]_D^{27}$ +15.2° (c=0.4, H_2O) (lit.,[3] $[\alpha]_D^{25}$ −19°, lit.,[5] $[\alpha]_D^{19}$ −12.6°, data for enantiomer of **7**). ^{1}H-NMR (400 MHz in D_2O) δ: 3.46 (1H, d, J=12.8 Hz, *trans*-5-H), 3.56 (1H, d, J=12.8, 3.7 Hz, *cis*-5-H), 4.02 (1H, s, 2-H), 4.26 (1H, d, J=3.7 Hz, 4-H), 4.47 (1H, s, 3-H). ^{13}C-NMR (100 MHz) δ: 51.80 (t), 68.41 (d), 74.83 (d), 79.22 (d), 172.05 (s). IR ν_{max}^{Nujol} cm^{-1}: 3512, 3260, 1618, 1576. MS *m/z*: 148 (MH$^+$). *Anal.* Calcd for $C_5H_9NO_4$: C, 40.82; H, 6.17; N, 9.52. Found: C, 40.89; H, 6.42; N, 9.42. Ninhydrin reaction: yellow.

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Chemistry of *O*-Silylated Ketene Acetals: A Mild and Convenient Synthesis of β-Lactam Antibiotics

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β-Amido sulfoxides (1) reacted with *O*-silylated ketene acetal (16) in dry acetonitrile in the presence of a catalytic amount of zinc iodide to give the 4-phenylthioazetidin-2-ones (17). Oxidation of 17 with *m*-chloroperbenzoic acid gave the corresponding sulfoxides (18), which were treated with 16 to give the azetidin-2-one esters (20), known precursors of PS-5-type carbapenem antibiotics.

Keywords *O*-silylated ketene acetal; intramolecular Pummerer-type reaction; 4-phenylthioazetidin-2-one; PS-5

Since the discovery of non-classical β-lactam antibiotics such as thienamycin and PS-5, great efforts have been made to find new methodologies and reagents suitable for the preparation of these naturally occurring carbapenem antibiotics.[1] Recently we described[2] a biomimetic approach to penicillin synthesis from the Arnstein tripeptide analogue by using our silicon-induced Pummerer-type reaction.[3] In connection with this study, we have briefly communicated[4] an application of the method to a synthesis of carbapenem antibiotics involving PS-5 from readily obtained β-amido sulfoxides (**1**). We now give a full account of this work.

Several known (**1a**,[5] **1d**[6]) and unknown sulfoxides (**1b**, **c** and **1e**—**h**) were prepared by the following routes as outlined in Chart 1. Sulfoxides (**1a**—**f**) were prepared from the appropriate α,β-unsaturated amides or esters. Saponification of the ester (**2**) with potassium hydroxide in ethanol after addition of thiophenol to methyl acrylate gave the acid (**3**).[7] Condensation of **3** with amines was performed either by the use of a powerful dehydrating agent, (trimethylsilyl)ethoxyacetylene,[8] or *via* the acid chloride intermediate to give the corresponding β-amido sulfides (**4**—**6**), which were oxidized with sodium periodate ($NaIO_4$) to give the corresponding β-amido sulfoxides (**1a**—**c**). 2-Methyl-β-amido sulfoxides (**1d**, **e**) were prepared from 2-methyl acrylamide *via* β-amido sulfides (**7** and **8**). Addition of thiophenol to 2-methyl acrylamide produced **7**, which was oxidized with $NaIO_4$ to give **1d**. Treatment of **7** with benzyl bromide in the presence of sodium hydride followed by oxidation with $NaIO_4$ gave **1e**. Other 2-ethyl-β-amido sulfoxides (**1f**—**h**) were prepared from ketene silyl acetal (**9**) in 3 or 4 steps. Treatment of **9** with chlorothioanisole (**10**) in the presence of titanium tetrachloride ($TiCl_4$) in methylene chloride (CH_2Cl_2) gave the ester (**11**), which was hydrolyzed with sodium hydroxide in methanol to give the acid (**12**). Condensation of **11** with amines in the presence of trimethylaluminum[9] gave the amides (**13** and **14**), which were oxidized with $NaIO_4$ to give **1f**, and **g** in good overall yields. Condensation of the acid (**12**) with diphenylmethylamine using (trimethylsilyl)ethoxyacetylene gave the amide (**15**), which was oxidized with $NaIO_4$ to give **1h**. All these compounds gave proton nuclear magnetic resonance (^1H-NMR), infrared (IR), and analytical data consistent with the expected structures.

Treatment of the β-amido sulfoxides (**1a**—**h**) with 1-(dimethyl-*tert*-butylsiloxy)-1-methoxyethylene (**16**) caused an intramolecular Pummerer-type reaction to give the corresponding 4-phenylthioazetidin-2-ones (**17a**—**h**) (Table I). A typical procedure is as follows. An excess of **16** was added to a solution of **1a** and a catalytic amount of zinc iodide (ZnI_2) in dry acetonitrile at room temperature, and the mixture was stirred for 1 h. After removal of the solvent, the residue was purified by column chromatography to give **17a** in good yield.[10] The generality of this reaction is indicated by the finding that both *N*-substituted (**1b**, **1c**, **1e**, **1g**, and **1h**) and *N*-unsubstituted

$CH_2{=}CHCO_2Me$ → **2**: R=Me; **3**: R=H (PhS–CH₂CH₂–C(=O)OR)

$CH_2{=}C(Me)CONH_2$ →

9 (Et–CH=C(OMe)(OSi^{t}BuMe$_2$)) —PhSCH₂Cl (**10**)→ **11**: R^3=Me; **12**: R^3=H

4: $R^1=R^2=H$
5: $R^1=H, R^2=CH_2Ph$
6: $R^1=H, R^2=CHPh_2$
7: $R^1=Me, R^2=H$
8: $R^1=Me, R^2=CH_2Ph$
13: $R^1=Et, R^2=H$
14: $R^1=Et, R^2=CH_2Ph$
15: $R^1=Et, R^2=CHPh_2$

1a: $R^1=R^2=H$
1b: $R^1=H, R^2=CH_2Ph$
1c: $R^1=H, R^2=CHPh_2$
1d: $R^1=Me, R^2=H$
1e: $R^1=Me, R^2=CH_2Ph$
1f: $R^1=Et, R^2=H$
1g: $R^1=Et, R^2=CH_2Ph$
1h: $R^1=Et, R^2=CHPh_2$

^{t}Bu = *tert*-Bu

Chart 1

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TABLE I. The Synthesis of β-Lactams (**17a—h**)

Run	Sulfoxide (1) R^1	Sulfoxide (1) R^2	Reaction conditions[a]	Product (17) R^3	Yield[b] (%)	Ratio[c] *cis* : *trans*
1	H	H **1a**	r.t. 1 h	Si^tBuMe_2 **17a**	88	
2	H	CH_2Ph **1b**	r.t. 6 h	CH_2Ph **17b**	73	
3	H	$CHPh_2$ **1c**	r.t. 3 h	$CHPh_2$ **17c**	93	
4	Me	H **1d**	r.t. 3 h	Bi^tBuMe_2 **17d**	77	72:28
5	Me	CH_2Ph **1e**	r.t.–65 °C 5 h	CH_2Ph **17e**	77	71:29
6	Et	H **1f**	r.t.–50 °C 14 h	Si^tBuMe_2 **17f**	75	63:37
7	Et	CH_2Ph **1g**	r.t. 1 d	CH_2Ph **17g**	63	59:41
8	Et	$CHPh_2$ **1h**	r.t.–50 °C 6 h	$CHPh_2$ **17h**	78	44:56

a) The reactions were carried out on 0.05—0.2 mmol scale of sulfoxides with 2—5 eq of **16** in the presence of a catalytic amount (0.05—0.1 eq) of ZnI_2. *b*) Isolated yields (by column chromatography on silica gel) are given. *c*) The ratios were determined by 500 MHz ^{1}H-NMR and HPLC. r.t. = room temperature.

Chart 2

Fig. 1

TABLE II. Substitution Reaction of **18a**

Entry	Catalyst	Solvent	Conditions	Yield (%)
1	ZnI_2	CH_3CN	r.t. 30 min	86
2	ZnI_2	CH_2Cl_2	r.t. 24 h	50
3	ZnI_2	THF	r.t. 4 h	60
4	$TiCl_4$, TMSOTf BF_3OEt_2, $SnCl_4$	CH_3CN	r.t. 15 min—18 h	Complex mixture
5	—	CH_3CN	r.t. 14 h—50 °C 3 d	0 (No reaction)

r.t: room temperature.

β-amido sulfides (**1a**, **1d**, and **1f**) reacted readily with **16** to give **17a—h** in high yields. These products were characterized on the basis of ^{1}H-NMR data and accurate mass spectra (MS); the *cis*/*trans* assignments (and ratios) for the 3,4-disubstituted azetidin-2-ones (**17d—h**) were made on the basis of 500 MHz ^{1}H-NMR spectrometric measurements and high performance liquid chromatography (HPLC) behavior.

Although a number of methods have been developed[1] for carbon–carbon bond formation at the C-4 position of azetidin-2-one, most of the methods start from 4-acetoxy and 4-chloroazetidin-2-ones and involve either strongly basic or acidic conditions or require low temperature. We found a versatile and practical method for carbon–carbon bond formation by using 4-phenylsulfinylazetidin-2-ones obtained from 4-phenylthioazetidin-2-ones. Oxidation of the sulfides (**17a**) with *m*-chloroperbenzoic acid (*m*-CPBA) in CH_2Cl_2 gave the sulfoxide (**18a**) (Chart 2), which was treated with **16** in the presence of a catalyst. Among various reaction conditions examined, the use of a catalytic amount of ZnI_2 in dry acetonitrile gave the best result (Table II). A typical experimental procedure is as follows for the formation of **19a** with **16**. A solution of **18a**, **16**, and a catalytic amount of ZnI_2 in dry acetonitrile was stirred at room temperature for 0.5 h. After usual work-up, a high yield of **19a** was obtained. Similarly, other 4-phenylsulfinylazetidin-2-ones (**18b**, **c** and **18f—h**) were reacted with **16** to give the corresponding azetidinone esters (**19b**, **c** and **19f—h**), which gave spectral and analytical data consistent with the expected structures. The reaction conditions and yields are summarized in Table III. In the case of 3-ethyl-4-phenylsulfinylazetidin-2-ones (**18f—h**), the *trans*-azetidin-2-one esters (**19f—h**) were produced selectively whether *cis*- or *trans*-azetidin-2-ones were used as the starting materials. Therefore, it is presumed that carbon–carbon bond formation in the reaction of 4-phenylsulfinylazetidin-2-ones with **16** proceeds *via* a nucleophilic attack of the ester enolate anion on the iminium intermediates (A) to give the *trans*-azetidin-2-one esters (**18f—h**) (Fig. 1). To our knowledge, this is the first example of the substitution of a sulfinyl group by an enol ester equivalent at the 4-position of azetidin-2-one.[11]

Finally, our attention was focused on the synthesis of a carbapenem antibiotic, PS-5. The *trans*-azetidin-2-one methyl ester (**19f**) was transesterified[12] with benzyl alcohol to give the *trans*-azetidin-2-one benzyl ester (**20**) in excellent yield. Desilylation of **20** with tetrabutylammonium fluoride (Bu_4NF) and acetic acid (AcOH) in tetrahydrofuran (THF)

TABLE III. Carbon–Carbon Bond Formation at the C-4 Position of Azetidin-2-ones

Run	Sulfoxide (18)	R^1	R^3	Reaction conditions[a)]	Product (19)	Yield[b)] (%)	Ratio[c)] *trans*: *cis*
1	**18a**	H	Si^tBuMe_2	r.t. 30 min	**19a**	86	
2	**18b**	H	CH_2Ph	r.t. 1 h	**19b**	52	
3	**18c**	H	$CHPh_2$	r.t. 10 min	**19c**	89	
4	**18f** (*cis*: *trans* = 63 : 37)	Et	Si^tBuMe_2	−20 °C 1 h	**19f**	79	94 : 6
5	**18f** (*cis*)	Et	Si^tBuMe_2	−20 °C 1 h	**19f**	73	95 : 5
6	**18f** (*trans*)	Et	Si^tBuMe_2	−20 °C 1 h	**19f**	77	95 : 5
7	**18g** (*cis*: *trans* = 59 : 41)	Et	CH_2Ph	−20 °C 10 min	**19g**	84	89 : 11
8	**18h** (*cis*: *trnas* = 44 : 56)	Et	$CHPh_2$	−20 °C 1 h	**19h**	80	91 : 9
9	**18i**	H	H	r.t. 30 min	**19a**[d)]	68	

a) The reactions were carried out on 0.05—0.2 mmol scale of sulfoxides with 2—4 eq of **16** in the presence of a catalytic amount (0.05—0.1 eq) of ZnI_2. *b*) Isolated yields (by column chromatography on silica gel) are given. *c*) The ratios were determined by 500 MHz ^{1}H-NMR. *d*) The *N*-silylated compound (**19a**) was obtained.

^{t}Bu = *tert*-Bu

Chart 3

followed by reductive debenzylation on 10% palladium–carbon (Pd-C) in ethanol gave the known *trans*-4-carboxy-3-ethylazetidin-2-one (**22**)[13] (60% overall yield), which is the key intermediate to PS-5. Furthermore, 3-ethyl-4-sulfinylazetidin-2-one (**18f**, *cis*/*trans* = 59/41) was treated with the silyl enol ether (**23**) to give the *trans*-azetidin-2-one diazo ester (**24**) in 43% yield. The ester (**24**) was treated with Bu_4NF and AcOH in THF to give the known desilylated diazo ester (**25**)[13,14] (92%), which is also the key intermediate to PS-5 · benzyl ester.

Experimental

All melting and boiling points are uncorrected. ^{1}H-NMR spectra were recorded on a Hitachi R-22 (90 MHz) or a JEOL JNM-GX 500 (500 MHz) spectrometer (with tetramethylsilane as an internal standard unless otherwise noted). IR absorption spectra were recorded on a JASCO HPIR-102 spectrophotometer. Low- and high-resolution MS were obtained with a JEOL JMS-D300 instrument, with a direct inlet system at 70 eV. For column chromatography, E. Merck silica gel (70—230 mesh ASTM) was used. For preparative thin layer chromatography (preparative TLC), E. Merck TLC plates precoated with Silica gel $60F_{254}$ (0.5 mm) were used.

O-Methyl-*O*-*tert*-butyldimethylsilyl Ketene Acetal (16) The ketene acetal (**16**) was prepared by the reported method.[15]

Methyl 3-(Phenylthio)propionate (2) A mixture of methyl acrylate (4.3 g, 50 mmol), thiophenol (5.5 g, 50 mmol), and triethylamine (0.5 ml) was stirred at room temperature for 5 min, then at 50—60 °C for 1 h. The reaction mixture was evaporated *in vacuo*. The residue was distilled under reduced pressure to give **2** (9.2 g, 94%) as a colorless oil, bp 108—110 °C (2 mmHg) (lit.[16] 113—115 °C (2 mmHg)).

3-(Phenylthio)propionic Acid (3) A solution of **2** (7.6 g, 38.5 mmol) in ethanol (50 ml) was added to a solution of potassium hydroxide (6.47 g) in water (50 ml). The mixture was refluxed for 1 h, cooled to room temperature, acidified with 10% hydrochloric acid, and extracted with CH_2Cl_2 (50 ml × 5). The combined CH_2Cl_2 layer was dried over Na_2SO_4. Evaporation of the solvent and crystallization of the residue gave the acid **3** (4.7 g, 67%) as colorless crystals, mp 58—59 °C (hexane) (lit.[7] 58—60 °C). IR ν_{max} ($CHCl_3$) cm^{-1}: 3600—2200, 1710. ^{1}H-NMR ($CDCl_3$) δ: 2.63 (2H, t, J = 7 Hz, O=CCH_2–), 3.13 (2H, t, J = 7 Hz, –CH_2SPh), 7.17—7.34 (5H, m, SPh), 8.40—9.00 (1H, br, COOH). MS m/z: 182 (M^+).

3-(Phenylthio)propionamide (4) A solution of **3** (204.7 mg, 1.12 mmol) and a catalytic amount of dimethylformamide in thionyl chloride (2.7 g, 22.7 mmol) was refluxed for 1 h and concentrated *in vacuo*. After the residue was cooled at 0 °C, 28% aqueous ammonium hydroxide was added. The mixture was stirred for 1 h, acidified with concentrated hydrochloric acid, and extracted with CH_2Cl_2 (20 ml × 5). The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, and dried over $MgSO_4$. Evaporation of the solvent *in vacuo* gave the residue, which was subjected to column chromatography on silica gel with AcOEt–hexane (1 : 3) to give **4** (127.3 mg, 63%) as colorless crystals, mp 119 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3540, 3420, 1685. ^{1}H-NMR ($CDCl_3$) δ: 2.47 (2H, t, J = 7 Hz, O=CCH_2–), 3.14 (2H, t, J = 7 Hz, –CH_2SPh), 7.1—7.4 (5H, m, SPh). *Anal.* Calcd for $C_9H_{11}NOS$: C, 59.63; H, 6.13; N, 7.73; S, 17.69. Found: C, 59.26; H, 6.03; N, 7.60; S, 17.45.

***N*-Benzyl-3-(phenylthio)propionamide (5)** (Trimethylsilyl)ethoxyacetylene (65.3 mg, 0.460 mmol) was added to a stirred solution of **3** (56 mg,

0.308 mmol), benzylamine (34.9 mg, 0.326 mmol) and HgO (3 mg, 0.0138 mmol) in $(CH_2Cl)_2$ (2 ml) at room temperature. The mixture was stirred at 60 °C for 8 h and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with CH_2Cl_2–Et_2O (20 : 1) to gave **5** (80 mg, 96%) as colorless crystals, mp 127 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3440, 1665. ^{1}H-NMR ($CDCl_3$) δ: 2.51 (2H, t, J=7 Hz, O=CCH_2–), 3.26 (2H, t, J=7 Hz, –$C\underline{H}_2$SPh), 4.41 (2H, d, J=6 Hz, –$C\underline{H}_2$Ph), 5.8—6.0 (1H, br, NH), 7.27 (10$\underline{H}$, m, ArH). MS m/z: 271 (M^+). *Anal.* Calcd for $C_{16}H_{17}NOS$: C, 70.80; H, 6.33; N, 5.16; S, 11.81. Found: C, 70.99; H, 6.37; N, 5.04; S, 11.60.

***N*-(1,1-Diphenylmethyl)-3-(phenylthio)propionamide (6)** In a similar fashion, **3** (100.7 mg, 0.553 mmol) was treated with diphenylmethylamine (126.5 mg, 0.691 mmol), (trimethylsilyl)ethoxyacetylene (160.0 mg, 1.13 mmol) and HgO (6.7 mg, 0.0309 mmol) in $(CH_2Cl)_2$ (5 ml) at 60 °C for 6 d to give **6** (194.1 mg, quant.) as colorless crystals, mp 131—133 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3445, 1665. ^{1}H-NMR ($CDCl_3$) δ: 2.50 (2H, t, J=7 Hz, O=CCH_2–), 3.20 (2H, t, J=7 Hz, –$C\underline{H}_2$SPh), 6.20 (1H, br, NH), 6.22 (1H, s, –$C\underline{H}Ph_2$), 7.22 (15H, m, ArH). MS m/z: 347 (M^+), 238 (M^+ – SPh). Exact MS Calcd for $C_{22}H_{21}NOS$: 347.1344. Found: 347.1346.

2-Methyl-3-(phenylthio)propionamide (7) Thiophenol (1.29 g, 11.7 mmol) and triethylamine (47.4 mg, 0.468 mmol) were added to a solution of 2-methylacrylamide (1.0 g, 11.7 mmol) in MeOH (10 ml) at 0 °C. The mixture was stirred at room temperature for 1 d and evaporated *in vacuo*. The residue was diluted with CH_2Cl_2 (50 ml), washed with 5% sodium hydroxide, water, and brine. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo* to give crude **7**, which was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (20 : 1) to give pure **7** (1.49 g, 65%) as colorless crystals, mp 73 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3540, 3500, 1680. ^{1}H-NMR ($CDCl_3$) δ: 1.28 (3H, d, J=7 Hz, –CH_3), 2.40—2.71 (1H, m, $CH_3C\underline{H}$<), 2.96 (1H, dd, J=6, 13 Hz, –C$\underline{H}$HSPh), 3.28 (1H, dd, J=7.5, 13 Hz, –CH$\underline{H}$SPh), 5.7—6.4 (2H, br, NH_2), 7.18—7.40 (5H, m, SPh). MS m/z: 195 (M^+). *Anal.* Calcd for $C_{10}H_{13}NOS$: C, 61.50; H, 6.72; N, 7.17; S, 16.42. Found: C, 61.42; H, 6.70; N, 7.05; S, 16.23.

***N*-Benzyl-2-methyl-3-(phenylthio)propionamide (8)** Compound **7** (199 mg, 1.02 mmol) was added to a suspension of NaH (60%, 47 mg, 1.18 mmol) in dry THF (3 ml) at –25 °C under nitrogen. After stirring of this mixture for 20 min, benzyl bromide (0.13 ml, 1.09 mmol) was added. The reaction mixture was stirred for 30 min under the same conditions and at room temperature for 2 h, and saturated with aqueous NH_4Cl. The aqueous layer was extracted with CH_2Cl_2 (20 ml × 5). The combined organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel with hexane–AcOEt (3 : 1) to give **8** (122 mg, 42%) as colorless crystals, mp 55—56 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450, 1665. ^{1}H-NMR ($CDCl_3$) δ: 1.28 (3H, d, J=7 Hz, –CH_3), 2.34—2.58 (1H, m, $CH_3C\underline{H}$<), 3.00 (1H, dd, J=6, 13 Hz, –C$\underline{H}$HSPh), 3.31 (1H, dd, J=8, 13 Hz, –CH$\underline{H}$SPh), 4.44 (2H, d, J=6 Hz, –CH_2Ph), 6.02 (1H, br, NH), 7.37 (10H, m, SPh). MS m/z: 285 (M^+). Exact MS Calcd for $C_{17}H_{19}NOS$: 285.1185. Found: 285.1185.

Methyl 2-(Phenylthiomethyl)butanoate (11) The ketene silyl acetal (**9**) (2.23 g, 10.3 mmol) was added to a stirred solution of chlorothioanisole (**10**, 1.36 g, 8.56 mmol) and $TiCl_4$ (1.73 g, 9.12 mmol) in CH_2Cl_2 (18 ml) at –78 °C under nitrogen. The mixture was stirred under the same conditions for 4.5 h, then partitioned between CH_2Cl_2 (50 ml) and water. The aqueous layer was extracted with CH_2Cl_2 (50 ml × 4). The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel with hexane–ether (20 : 1) to give **11** (1.16 g, 60% from **10**) as a colorless oil, bp 90—95 °C (0.25 mmHg) (bath temperature). IR ν_{max} ($CHCl_3$) cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7 Hz, CH_3), 1.66 (2H, q, J=7 Hz, –$C\underline{H}_2CH_3$), 2.56 (1H, m, –$C\underline{H}CH_2$SPh), 2.99 (1H, dd, J=6, 13 Hz, –C$\underline{H}$HSPh), 3.18 (1H, dd, J=8, 13 Hz, –CH$\underline{H}$SPh), 3.67 (3H, s, –OCH_3), 7.12 (5H, m, SPh). MS m/z: 224 (M^+).

2-(Phenylthiomethyl)butanoic acid (12) A solution of sodium hydroxide (213 mg, 8.875 mmol) in water (2 ml) was added to a stirred solution of **11** (42.9 mg, 0.191 mmol) in methanol (2 ml). The mixture was refluxed for 1.5 h, poured into water (20 ml), acidified with 10% hydrochloric acid, and extracted with CH_2Cl_2 (20 ml × 4). The combined organic layer was washed with water, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel with CH_2Cl_2–MeOH (10 : 1) to give **12** (25.8 mg, 64%) as a colorless oil, bp 80—85 °C (0.15 mmHg, bath temperature). IR ν_{max} ($CHCl_3$) cm^{-1}: 3600—2400, 1710. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, J=7 Hz, –CH_3), 1.75 (2H, quint, J=7 Hz, –$C\underline{H}_2CH_3$), 2.56 (1H, m, –$C\underline{H}CH_2$SPh), 2.98 (1H, dd, J=6, 14 Hz, C$\underline{H}$HSPh), 3.18 (1H, dd, J=8, 14 Hz, –CH$\underline{H}$SPh), 7.20—7.38 (5H, m, SPh), 9.67 (1H, br, COOH). MS m/z: 210 (M^+).

2-(Phenylthiomethyl)butanamide (13) A 1.0 M solution of trimethylaluminum in hexane (4.5 ml, 4.5 mmol) was added to a stirred suspension of ammonium chloride (238.5 mg, 4.46 mmol) in dry benzene (2 ml) at 0 °C under nitrogen. The mixture was stirred at 0 °C for 20 min and at room temperature for 45 min. Compound **11** (200.0 mg, 0.893 mmol) was added to the mixture, which was refluxed for 3 h and cooled to 0 °C. Then 5% hydrochloric acid was added to decompose excess trimethylaluminum, and the mixture was extracted with CH_2Cl_2 (20 ml × 5). The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel with CH_2Cl_2–MeOH (25 : 1) to give **13** (91.4 mg, 49%) as colorless crystals, mp 79—80 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3540, 3420, 1680. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=7 Hz, –CH_3), 1.63 (2H, q, J=7 Hz, –$C\underline{H}_2CH_3$), 2.27 (1H, m, –$C\underline{H}CH_2$SPh). 2.97 (1H, dd, J=5.5, 12 Hz, –C$\underline{H}$HSPh), 3.18 (1H, dd, J=8, 12 Hz, –CH$\underline{H}$SPh), 5.45—6.10 (2H, br, NH_2), 7.11—7.33 (5H, m, SPh). MS m/z: 209 (M^+). Exact MS Calcd for $C_{11}H_{15}NOS$: 209.0874. Found: 209.0875.

***N*-Benzyl-2-(phenylthiomethyl)butanamide (14)** A 1.0 M solution of trimethylalumium in hexane (7.4 ml, 7.4 mmol) was added to a stirred solution of benzylamine (789.0 mg, 7.37 mmol) in dry benzene (2 ml) at –10 °C under nitrogren. The mixture was stirred at –10 °C for 20 min and at room temperature for 45 min. Compound **11** (509.0 mg, 2.27 mmol) was added, and the whole was refluxed for 2 h and cooled to 0 °C. Then 5% hydrochloric acid was added to decompose excess trimethylaluminum, and the solution was extracted with CH_2Cl_2 (30 ml × 4). The combined organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel with AcOEt–hexane (4 : 1) to give **14** (556.0 mg, 82%) as colorless crystals, mp 87—88 °C (AcOEt–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3440, 1665. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=7 Hz, –CH_3), 1.61—1.83 2H, m, –$C\underline{H}_2CH_3$), 2.14—2.33 (1H, m, –$C\underline{H}CH_2$SPh), 3.07 (1H, dd, J=5.5, 13.5 Hz, –C$\underline{H}$HSPh), 3.29 (1H, dd, J=8.5, 13.5 Hz, –CH$\underline{H}$SPh), 4.49 (2H, d, J=6 Hz, –$C\underline{H}_2$Ph), 5.87 (1H, br, NH), 7.26—7.38 (10H, m, ArH). MS m/z: 299 (M^+). *Anal.* Calcd for $C_{18}H_{21}NOS$: C, 72.20; H, 7.07; N, 4.08; S, 10.70. Found: C, 72.20; H, 7.03; N, 4.57; S, 10.68.

***N*-(1,1-Diphenylmethyl)-2-(phenylthiomethyl)butanamide (15)** (Trimethylsilyl)ethoxyacetylene (108.3 mg, 0.763 mmol) was added to a stirred solution of **12** (77.9 mg, 0.371 mmol), diphenylmethylamine (81.5 mg, 0.445 mmol) and HgO (6.4 mg, 0.0295 mmol) in $(CH_2Cl)_2$ (4 ml) at room temperature. The mixture was stirred at 60 °C for 7 d and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with hexane–AcOEt (6 : 1) to give **15** (118.3 mg, 85%) as colorless crystals, mp 87—89 °C ($CHCl_3$–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450, 1670. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7 Hz, –CH_3), 1.66 (2H, q, J=7 Hz, –CH_2CH_3), 2.23 (1H, m, –$C\underline{H}CH_2$SPh), 3.03 (1H, dd, J=5.5, 13 Hz, –C$\underline{H}$HSPh), 3.15 (1H, dd, J=8.5, 13 Hz, –CH$\underline{H}$SPh), 6.09 (1H, br d, J=8 Hz, NH), 6.27 (1H, d, J=8 Hz, –$C\underline{H}Ph_2$), 7.19—7.24 (15H, m, ArH). MS m/z: 375 (M^+). *Anal.* Calcd for $C_{24}H_{25}NOS$: C, 76.76; H, 6.71; N, 3.73; S, 8.54. Found: C, 76.51; H, 6.71; N, 3.75; S, 8.16.

General Procedure for the Preparation of β-Amido Sulfoxides (1a—h) $NaIO_4$ (1.5 mmol) was added to a stirred solution of a sulfide (**4**—**8** or **13**—**15**, 1 mmol) in MeOH (10 ml). The mixture was stirred at room temperature overnight and evaporated *in vacuo*. The residue was partitioned between CH_2Cl_2 (20 ml) and water (20 ml), and then the aqueous layer was extracted with CH_2Cl_2 (20 ml × 4). The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was subjected to column chromatography or preparative TLC on silica gel with CH_2Cl_2–MeOH, AcOEt to give the corresponding sulfoxide.

3-(Phenylsulfinyl)propionamide (1a) This (61.7 mg, 93%) was prepared from **4** (60.4 mg, 0.336 mmol) and $NaIO_4$ (117.5 mg, 0.549 mmol) in MeOH (3 ml) as colorless crystals, mp 135 °C (MeOH–Et_2O–hexane), (lit.[6] 129—130.5 °C). The IR and ^{1}H-NMR spectral data of **1a** were identical with those of lit.[6]

***N*-Benzyl-3-(phenylsulfinyl)propionamide (1b)** This (68.3 mg, quant.) was prepared from **5** (65 mg, 0.217 mmol) and $NaIO_4$ (70 mg, 0.327 mmol) in MeOH (3 ml) as colorless crystals, mp 94 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3445, 1665, 1035. ^{1}H-NMR ($CDCl_3$) δ: 2.24—3.39 (4H, m, –$C\underline{H}_2C\underline{H}_2$S(O)Ph), 4.42 (2H, d, J=6 Hz, –$C\underline{H}_2$Ph), 7.32 (5H, s,

$-CH_2Ph$), 7.58 (5H, s, –S(O)Ph). MS *m*/*z*: 287 (M^+). *Anal.* Calcd for $C_{16}H_{17}NO_2S$: C, 66.86; H, 5.97; N, 4.87; S, 11.6. Found: C, 66.56; H, 5.98; N, 4.73; S, 11.01.

***N*-(1,1-Diphenylmethyl)-3-(phenylsulfinyl)propionamide (1c)** This (48.2 mg, 86%) was prepared from **6** (53.6 mg, 0.154 mmol) and $NaIO_4$ (49.6 mg, 0.231 mmol) in MeOH (3 ml) as colorless crystals, mp 157—158 °C ($CHCl_3$–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3440, 1665, 1035. ^{1}H-NMR ($CDCl_3$) δ: 2.44—3.34 (4H, m, $-CH_2CH_2S(O)Ph$), 6.17 (1H, d, *J*=8 Hz, $CHPh_2$), 7.18 (10H, s, $CHPh_2$), 7.43 (5H, s, S(O)Ph). MS *m*/*z*: 363 (M^+). Exact MS Calcd for $C_{22}H_{21}NO_2S$: 363.1293. Found: 363.1313.

2-Methyl-3-(phenylsulfinyl)propionamide (1d) This (107.3 mg, 96%) was prepared from **7** (104 mg, 0.53 mmol) and $NaIO_4$ (145 mg, 0.68 mmol) in MeOH (5 ml) as colorless crystals, mp 99—105 °C (CH_2Cl_2–hexane) (lit.[6]) 108—110 °C). IR ν_{max} (KBr) cm^{-1}: 3350, 3200, 1675, 1020. ^{1}H-NMR ($CDCl_3$) δ: 1.23, 1.44 (total 3H, each d, *J*=7 Hz, CH_3–), 2.5—3.9 (total 3H, m, $>CHCH_2SPh$), 6.04, 6.17, 6.78, 7.03 (total 2H, each brs, NH_2), 7.5 (5H, m, S(O)Ph). (The signals indicated this product to be a 1 : 1 mixture of geometrical isomers). MS *m*/*z*: 211 (M^+).

***N*-Benzyl-2-methyl-3-(phenylsulfinyl)propionamide (1e)** This (103 mg, 86%) was prepared from **8** (113 mg, 0.40 mmol) and $NaIO_4$ (110 mg, 0.514 mmol) in MeOH (2 ml) as colorless crystals, mp 112—122 °C (CH_2Cl_2–hexane). IR ν_{max}($CHCl_3$) cm^{-1}: 3450, 1665, 1040. ^{1}H-NMR ($CDCl_3$) δ: 1.23—1.42 (total 3H, each d, *J*=7 Hz, CH_3–), 2.78—3.40 (total 3H, m, $>CHCH_2SPh$), 4.28, 4.30 (1/2×2H, each d, *J*=5.5 Hz, $-CH_2Ph$), 4.49 (1/2×2H, d, *J*=5.5 Hz, $-CH_2Ph$), 6.70—6.95 (1H, br, NH), 7.20, 7.27 (total 5H, each s, CH_2Ph), 7.49 (5H, s, S(O)Ph). The signals indicated this product to be a 1 : 1 mixture of geometrical isomers). MS *m*/*z*: 301 (M^+). *Anal.* Calcd for $C_{17}H_{19}NO_2S$: C, 67.73 H, 6.37; N, 4.65; S, 10.64. Found: C, 67.65; H, 6.41; N, 4.52; S, 10.47.

2-(Phenylsulfinylmethyl)butanamide (1f) This (51.7 mg, quant.) was prepared from **13** (45.3 mg, 0.217 mmol) and $NaIO_4$ (69.6 mg, 0.325 mmol) in MeOH (3 ml) as colorless crystals, mp 120—128 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3530, 3410, 1680, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.88, 1.00 (total 3H, each t, *J*=7.5 Hz, $-CH_2CH_3$), 1.6—2.0 (2H, m, $-CH_2CH_3$), 2.6—3.2 (total 3H, m, $>CHCH_2S(O)Ph$), 5.5—6.0 (2H, br, NH_2), 7.3—7.6 (5H, m, S(O)Ph). The signals indicated this product to be a mixture of geometrical isomers). MS *m*/*z*: 225 (M^+). Exact MS Calcd for $C_{11}H_{15}NO_2S$: 225.0824. Found: 225.0825.

***N*-Benzyl-2-(phenylsulfinylmethyl)butanamide (1g)** This (12 mg, 95%) was prepared from **14** (12 mg, 0.04 mmol) and $NaIO_4$ (20 mg, 0.093 mmol) in MeOH (1 ml) as colorless crystals, mp 125—130 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3440, 1665, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.92, 1.00 (total 3H, each d, *J*=7 Hz, $-CH_2CH_3$), 1.7—2.1 (2H, m, $-CH_2CH_3$), 2.6—2.9 (1H, br, EtCH<), 2.9—3.3 (2H, m, $-CH_2SPh$), 4.32, 4.41, 4.59 4.61 (total 2H, each d, *J*=6 Hz, $-CH_2Ph$), 6.70 (1H, br, NH), 7.36—7.41 (5H, m, CH_2Ph), 7.60—7.64 (5H, s, S(O)Ph). (The signals indicated this product to be a mixture of geometrical isomers). MS *m*/*z*: 315 (M^+). Exact MS Calcd for $C_{18}H_{21}NO_2S$: 315.1290. Found: 315.1265.

***N*-(1,1-Diphenylmethyl)-2-(phenylsulfinylmethyl)butanamide (1h)** This (57.7 mg, 65%) was prepared from **15** (85.4 mg, 0.228 mmol) and $NaIO_4$ (73.1 mg, 0.342 mmol) in MeOH (3 ml) as colorless crystals, mp 175—176 °C ($CHCl_3$–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 3445, 1665, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.87, 0.97 (total 3H, t, *J*=7 Hz, $-CH_2CH_3$), 1.47—2.01 (2H, m, $-CH_2CH_3$), 2.54—3.33 (total 3H, m, EtCH<, $-CH_2S(O)Ph$), 6.14, 6.32 (total 1H, each d, *J*=8 Hz, $-CHPh_2$), 7.18, 7.24 (total 10H, each s, $CHCPh_2$), 7.38—7.42 (5H, m, S(O)Ph). (The signals indicated this product to be a mixture of geometrical isomers). MS *m*/*z*: 391 (M^+), 390 (M^+-1), 376 (M^+-Me).

General Procedure for the Reaction of β-Amido Sulfoxides (1a—h) with the Ketene Silyl Acetal (16) The ketene silyl acetal (**16**, 3—5 mmol) was added to a stirred solution of β-amido sulfoxide (**1**, 1 mmol) and ZnI_2 (0.05—0.1 mmol) in dry CH_3CN (10 ml) at room temperature under nitrogen. The mixture was stirred at the temperature and for the period indicated in Table I, then partitioned between CH_2Cl_2 (20 ml) and saturated aqueous $NaHCO_3$ (20 ml). The aqueous layer was extracted with CH_2Cl_2 (20 ml×4). The combined extract was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography or preparative TLC on silica gel with hexane–AcOEt to give the cyclized product.

***N*-(*tert*-Butyldimethylsilyl)-4-(phenylthio)azetidin-2-one (17a)** This (134.8 mg, 0.406 mmol) was obtained from **1a** (102.5 mg, 0.520 mmol), **16** (293.5 mg, 1.56 mmol), and ZnI_2 (8.3 mg, 0.026 mmol) in CH_3CN (5 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1740. ^{1}H-NMR ($CDCl_3$) δ: 0.31, 0.32 (total 6H, each s, Me_2Si), 1.00 (9H, s, *tert*-BuSi), 3.03 (1H, dd, *J*=2, 15 Hz, –CHHCO), 3.51 (1H, dd, *J*=5, 15 Hz, –CHHCO), 4.90 (1H, dd, *J*=2, 5 Hz, >CHSPh), 7.24—7.42 (5H, m, SPh). Exact MS Calcd for $C_{15}N_{23}NOSSi$: 293.1268. Found: 293.1243.

***N*-Benzyl-4-(phenylthio)azetidin-2-one (17b)** This (61.4 mg, 73%) was obtained from **1b** (90.0 mg, 0.314 mmol), **16** (300 mg, 0.60 mmol), and ZnI_2 (13 mg, 0.041 mmol) in CH_3CN (2 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1750. ^{1}H-NMR ($CDCl_3$) δ: 2.89 (1H, dd, *J*=2, 15 Hz, –CHHCO), 3.32 (1H, dd, *J*=5, 15 Hz, –CHHCO), 4.13 4.79 (total 2H, each d, *J*=15 Hz, CH_2Ph), 4.83 (1H, dd, *J*=2, 5 Hz, >CHSPh), 7.16—7.42 (10H, m, ArH). Exact MS Calcd for $C_{16}H_{15}NOS$: 269.0875. Found: 269.0882.

***N*-(1,1-Diphenymethyl)-4-(phenylthio)azetidin-2-one (17c)** This (26.0 mg, 93%) was obtained from **1c** (29.4 mg, 0.08 mmol), **16** (48 mg, 0.255 mmol), and ZnI_2 (13 mg, 0.041 mmol) in CH_3CN (2 ml) as colorless crystals, mp 90 °C ($CHCl_3$–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 1775. ^{1}H-NMR ($CDCl_3$) δ: 2.94 (1H, dd, *J*=2.5, 15 Hz, –CHHCO), 3.28 (1H, dd, *J*=4.5, 15Hz, –CHHCO), 4.79 (1H, dd, *J*=2.5 H, 5 Hz, >CHSPh), 5.71 (1H, s, $CHPh_2$), 7.20, 7.22, 7.27 (15H, each s, ArH). Exact MS Calcd for $C_{22}H_{19}NOS$: 345.1187. Found: 345.1190.

***N*-(*tert*-Butyldimethylsilyl)-3-methyl-4-(phenylsulfinyl)azetidin-2-one-(17d)** This (66.7 mg, 77%, *cis*:*trans*=72:28) was obtained from **1d** (59.4 mg, 0.282 mmol), **16** (309.3 mg, 1.65 mmol), and ZnI_2 (9 mg, 0.028 mmol) in CH_3CN as a colorless oil; ^{1}H-NMR and HPLC showed *cis*:*trans*=72:28. The pure isomers (*cis*-**17d** and *trans*-**17d**) were isolated by column chromatography for characterization. *cis*-**17d**: a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$) δ: 0.27 (6H, s, Me_2Si), 0.98 (9H, s, *tert*-Bu), 1.36 (3H, d, *J*=8 Hz, CH_3C<), 3.69 (1H, qd, *J*=8, 5 Hz, >CHCO), 5.04 (1H, d, *J*=5 Hz, >CHSPh), 7.24 (5H, m, –SPh). MS *m*/*z*: 307 (M^+). *trans*-**17d**: a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$) δ: 0.29 (6H, s, Me_2Si), 0.98 (9H, s, *tert*-Bu), 1.27 (3H, d, *J*=8 Hz, CH_3C<), 3.19 (1H, qd, *J*=8, 2 Hz, >CHCO), 4.49 (1H, d, *J*=2 Hz, >CHSPh), 7.18—7.40 (5H, m, –SPh). MS *m*/*z*: 307 (M^+).

***N*-Benzyl-3-methyl-4-(phenylthio)azetidin-2-one (17e)** This (22.2 mg, 77%, *cis*:*trans*=71:29) was obtained from **1e** (31.0 mg, 0.103 mmol), **16** (24 mg, 0.128 mmol),, and ZnI_2 (6 mg, 0.0188 mmol) in CH_3CN (1 ml) as a colorless oil; ^{1}H-NMR and HPLC showed *cis*:*trans*=71:29. The pure isomers (*cis*-**17e** and *trans*-**17e**) were isolated by column chromatography for characterization. *cis*-**17e**: a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 1.44 (3H, d, *J*=8 Hz, $-CH_3C$<), 3.64 (1H qd, *J*=8, 5 Hz, >CHCO), 4.09, 4.78 (total 2H, each d, *J*=15 Hz, CH_2Ph), 5.03 (1H, d, *J*=5 Hz, >CHSPh), 7.09—7.40 (10H, m, ArH). Exact MS Calcd for $C_{17}H_{17}NOS$; 283.1029. Found: 283.1009. *trans*-**17e**: a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (3H, d, *J*=8 Hz, CH_3C<), 3.02—3.28 (1H, m, >CHCO), 4.44 (1H, d, *J*=2 Hz, >CHSPh), 4.11, 4.88 (total 2H, each d, *J*=15 Hz, CH_2Ph), 7.19—7.42 (10H, m, ArH). Exact MS Calcd for $C_{17}H_{17}NOS$: 283.1030. Found: 283.1005.

***N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(phenylthio)azetidin-2-one (17f, *cis*:*trans*=63:37)** This (159.3 mg, 75%, *cis*:*trans*=63:37) was obtained from **1f** (150.0 mg, 0.667 mmol), **16** (377.4 mg, 2.01 mmol), and ZnI_2 (10.0 mg, 0.0313 mmol) in CH_3CN (4 ml) as a colorless oil; ^{1}H-NMR and HPLC showed *cis*:*trans*=63:37. The pure isomers (*cis*-**17f** and *trans*-**17f**) were isolated by column chromatography for characterization.

(3*S**,4*S**)-*N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(phenylthio)azetidin-2-one (*cis*-**17f**): a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1735, 1580. ^{1}H-NMR ($CDCl_3$) δ: 0.23, 0.26 (total 6H, each s, Me_2Si), 0.98 (9H, s, *tert*-BuSi), 1.07 (3H, t, *J*=7 Hz, $-CH_2CH_3$), 1.70—2.00 (2H, m, $-CH_2CH_3$), 3.47 (1H, td, *J*=7.5, 5 Hz, >CHCO), 5.03 (1H, d, *J*=5 Hz, >CHSPh), 7.13—7.31 (5H, m, SPh). Exact MS Calcd for $C_{17}H_{27}NOSSi$: 321.1583. Found: 321.1589.

(3*S**,4*R**)-*N*-(*tert*-Butyldimethysilyl)-3-ethyl-4-(phenylthio)azetidin-2-one (*trans*-**17f**): a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1735, 1580. ^{1}H-NMR ($CDCl_3$) δ: 0.18 (6H, s, Me_2Si), 0.87 (3H, t, *J*=7 Hz, $-CH_2CH_3$), 0.99 (9H, s, *tert*-BuSi), 1.60—1.87 (2H, m, $-CH_2CH_3$), 3.13 (1H, td, *J*=7.5, 2 Hz, >CHCO), 4.56 (1H, d, *J*=2 Hz, >CHSPh), 7.20—7.37 (5H, m, SPh). Exact MS Calcd for $C_{17}H_{27}NOSSi$: 321.1579. Found: 321.1571.

***N*-Benzyl-3-ethyl-4-(phenylthio)azetidin-2-one (17g)** This (74.7 mg, 63%, *cis*:*trans*=59:41) was obtained from **1g** (125.8 mg, 0.399 mmol), **16** (384 mg, 2.04 mmol), and ZnI_2 (17.0 mg, 0.0533 mmol) in CH_3CN (2 ml) as a pale yellow oil; ^{1}H-NMR and HPLC showed *cis*:*trans*=59:41. The pure isomers (*cis*-**17g** and *trans*-**17g**) were isolated by column chromatography for characterization *cis*-**17g**: a pale yellow oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, t, *J*=7 Hz, $-CH_2CH_3$), 1.81 (2H, m, $-CH_2CH_3$), 3.34 (1H, dt, *J*=5, 7 Hz, >CHCO), 3.99, 4.68 (total 2H, each d, *J*=15 Hz, $-CH_2Ph$), 4.90 (1H, d, *J*=5 Hz, >CHSPh), 6.9—7.4 (10H, m, ArH). Exact MS Calcd for $C_{18}H_{19}NOS$: 297.1185. Found: 297.1179. *trans*-**17g**: colorless crystals, mp 45—46 °C

(CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, J=7 Hz, –$CH_2C\underline{H}_3$), 1.6—2.0 (2H, m, –$C\underline{H}_2CH_3$), 3.0 (1H, dt, J=2, 7Hz, >CHCO), 4.02, 4.73 (total 2H, each d, J=15 Hz, –$C\underline{H}_2$Ph), 4.45 (1H, d, J=2 Hz, >C$\underline{H}$SPh), 6.9—7.4 (10H, m, ArH). Exact MS Calcd for $C_{18}H_{19}NOS$: 297.1185. Found: 297.1177.

***N*-(1,1-Diphenymethyl)-3-ethyl-4-(phenylthio)azetidin-2-one (17h)** This (38.3 mg, 78%, *cis*:*trans*=44:56) was obtained from **1h** (51.4 mg, 0.131 mmol), **16** (124.3 mg, 0.661 mmol), and ZnI_2 (3.8 mg, 0.012 mmol) in CH_3CN (3 ml) as a pale yellow oil; ^{1}H-NMR and HPLC showed *cis*:*trans*=44:56. The pure isomers (*cis*-**17h** and *trans*-**17h**) were isolated by column chromatography for characterization. *cis*-**17h**: a yellow oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1745 ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7 Hz, –$CH_2C\underline{H}_3$), 1.9 (2H, m, –$C\underline{H}_2CH_3$), 3.32 (1H, td, J=7, 4.5, >CHCO), 4.92 (1H, d, J=4.5 Hz, >C$\underline{H}$SPh), 5.76 (1H, s, C$\underline{H}$$Ph_2$), 7.0—7.4 (15H, m, ArH). Exact MS Calcd for $C_{24}H_{23}NOS$ – SPh: 264.1388. Found: 264.1393. *trans*-**17h**: colorless crystals, mp 83—84 °C (CH_2Cl_2–hexane). IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7 Hz, –$CH_2C\underline{H}_3$), 1.5—1.9 (2H, m, –$C\underline{H}_2CH_3$), 3.09 (1H, td, J=7, 2 Hz, >CHCO), 4.48 (1H, d, J=2 Hz, >C$\underline{H}$SPh), 5.71 (1H, s, C$\underline{H}$$Ph_2$), 7.0—7.4 (15 H, m, ArH). Exact MS Calcd for $C_{24}H_{23}$ NOS – SPh: 264.1389. Found: 264.1389.

General Procedure for the Preparation of 4-Phenylsulfinylazetidin-2-one (18a—c, f—h) *m*-CPBA (80%, 1 mmol) was added to a stirred solution of 4-phenylthioazetidin-2-one (**18a—c** or **18f—h**, 1 mmol) in CH_2Cl_2 (3 ml) at 0 °C for 30 min. The reaction mixture was diluted with CH_2Cl_2 (50 ml) and washed with saturated aqueous $NaHCO_3$ (30 ml). The aqueous layer was extracted with CH_2Cl_2 (20 ml × 4). The combined organic extract was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography or preparative TLC on silica gel with hexane–AcOEt to give the corresponding sulfoxide.

***N*-(*tert*-Butyldimethylsilyl)-4-(phenylsulfinyl)azetidin-2-one (18a)** This (24.7 mg, quant.) was obtained from **17a** (23.3 mg, 0.0795 mmol), *m*-CPBA (80%, 17.1 mg, 0.0795 mmol) in CH_2Cl_2 (3 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1760, 1045. ^{1}H-NMR ($CDCl_3$) δ: 0.38, 0.40, 0.42, 0.43 (total 6H, each s, Me_2Si), 1.06 (9H, s, *tert*-BuSi), 2.70 (1/2 × 1H, dd, J=5, 15.5 Hz, –C$\underline{H}$HCO), 2.79 (1/2 × 1H, dd, J=3, 16.5 Hz, –CH$\underline{H}$CO), 3.10 (1/2 × 1H, dd, J=5.5, 16.5 Hz, >CH$\underline{H}$CO), 3.60 (1/2 × 1H, dd, J=2, 15.5 Hz, –CH$\underline{H}$CO), 4.29 (1/2 × 1H, dd, J=2, 5 Hz, >CHSPh), 4.43 (1/2 × 1H, dd, J=2, 5.5 Hz, >C$\underline{H}$SPh), 7.49—7.67 (5H, m, SPh). Exact MS Calcd for $C_{15}H_{23}NO_2SSi$ – *tert*-Bu: 252.0512. Found: 252.0511.

***N*-Benzyl-4-(phenylsulfinyl)azetidin-2-one (18b)** This (45.9 mg, 93%) was obtained from **17b** (46.9 mg, 0.174 mmol), *m*-CPBA (80%, 37.6 mg, 0.174 mmol) in CH_2Cl_2 (3 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1765, 1445, 1375, 1085, 1040. ^{1}H-NMR ($CDCl_3$) δ: 2.65 (1H, dd, J=5, 15 Hz, –C$\underline{H}$HCO), 3.42 (1H, dd, J=2, 15 Hz, –CH$\underline{H}$CO), 4.17 (1H, dd, J=2, 5 Hz, >C$\underline{H}$SPh), 4.29, 4.76 (total 2H, each d, J=15 Hz, C$\underline{H}_2$Ph), 7.22—7.56 (10H, m, ArH). Exact MS Calcd for $C_{16}H_{15}NO_2S$ – S(O)Ph: 160.0760. Found: 160.0757.

***N*-(1,1-Diphenymethyl)-4-(phenylsulfinyl)azetidin-2-one (18c)** This (16.2 mg, 67%) was obtained from **17c** (23.1 mg, 0.067 mmol), *m*-CPBA (80%, 15.1 mg, 0.0704 mmol) in CH_2Cl_2 (1 ml) as colorless crystals. IR ν_{max} ($CHCl_3$) cm^{-1}: 1760, 1050. ^{1}H-NMR ($CDCl_3$) δ: 2.63 (68/100 × 1H, dd, J=5, 15 Hz, –C$\underline{H}$HCO), 2.99 (32/100 × 2H, d, J=4 Hz, –$C\underline{H}_2$CO), 3.63 (68/100 × 1H, dd, J=2, 15 Hz, –CH$\underline{H}$CO), 4.13 (1H, dd, J=2.5, 5 Hz, >C$\underline{H}$SPh), 4.53 (32/100 × 1H, t, J=5 Hz, >C$\underline{H}$SPh), 5.68 (32/100 × 1H, s, C$\underline{H}$$Ph_2$), 6.18 (68/100 × 1H, s, C$\underline{H}$$Ph_2$), 7.40 (15H, each s, ArH). Exact MS Calcd for $C_{22}H_{19}NO_2S$ – S(O)Ph: 236.1017. Found: 236.1073.

***N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(phenylsulfinyl)azetidin-2-one (18f, *cis*:*trans*=63:37)** This (21.3 mg, 85%, *cis*:*trans*=63:37) was obtained from **17f** (*cis*:*trans*=63:37, 24 mg, 0.0748 mmol), *m*-CPBA (80%, 16.1 mg, 0.0748 mmol) in CH_2Cl_2 (2 ml) as a colorless oil.

(3*S,4*S**)-*N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(phenylsulfinyl)azetidin-2-one (*cis*-18f)** This (*cis*, 11.7 mg, 81%) was obtained from *cis*-**17f** (13.7 mg, 0.0427 mmol), *m*-CPBA (80%, 9.6 mg, 0.0448 mmol) in CH_2Cl_2 (1.5 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1750, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.22, 0.29 (total 1/3 × 6H each, s, Me_2Si), 0.26, 0.31 (total 2/3 × 6H each, s, Me_2Si), 0.82 (3H, t, J=7 Hz, –$CH_2C\underline{H}_3$), 1.02 (1/3 × 9H, s, *tert*-BuSi), 1.04 (2/3 × 9H, s, *tert*-BuSi), 1.27—1.78 (2/3 × 2H, m, –$C\underline{H}_2CH_3$), 2.18—2.51 (1/3 × 2H, m, –$C\underline{H}_2CH_3$), 3.24—4.73 (total 1H, m, >CHCO), 4.42 (2/3 × 1H, d, J=5.5 Hz, >C$\underline{H}$SPh), 4.62 (1/3 × 1H, d, J=5.5 Hz, >C$\underline{H}$SPh), 7.44—7.62 (5H, m, S(O)Ph). MS m/z: 322 (M^+ – Me), 280 (M^+ – *tert*-Bu). Exact MS Calcd for $C_{17}H_{27}NO_2SSi$ – *tert*-Bu: 280.0824. Found: 280.0819.

(3*S,4*R**)-*N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(phenylsulfinyl)azetidin-2-one (*trans*-18f)** This (*trans*, 5.7 mg, 99%) was obtained from *trans*-**17f** (5.5 mg, 0.0171 mmol), *m*-CPBA (80%, 3.7 mg, 0.0171 mmol) in CH_2Cl_2 (1 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1750, 1030. ^{1}H-NMR ($CDCl_3$) δ: 0.33, 0.38. 0.40 (total 6H, each s, Me_2Si), 0.53—0.71 (total 3H, m, –$CH_2C\underline{H}_3$), 1.04 (9H, s, *tert*-BuSi), 1.18—1.49 (2H, m, –$C\underline{H}_2CH_3$), 2.91 (1/2 × 1H, td, J=7, 2 Hz, >CHCO), 3.69 (1/2 × 1H, td, J=7, 2 Hz, >CHCO), 4.00 (1/2 × 1H, d, J=2 Hz, >C$\underline{H}$SPh), 4.13 (1/2 × 1H, d, J=2 Hz, >C$\underline{H}$SPh), 7.47—7.62 (5H, m, S(O)Ph). MS m/z: 322 (M^+ – Me), 280 (M^+ – *tert*-Bu). Exact MS Calcd for $C_{17}H_{27}NO_2SSi$ – *tert*-Bu: 280.0828. Found: 280.0838.

***N*-Benzyl-3-ethyl-4-(phenylsulfinyl)azetidin-2-one (18g)** This (7.6 mg, 53%, *cis*:*trans*=59:41) was obtained from **17g** (13.6 mg, 0.0458 mmol), *m*-CPBA (80%, 9.9 mg, 0.0458 mmol) in CH_2Cl_2 (1 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1755, 1035. ^{1}H-NMR ($CDCl_3$) δ: 1.18—1.33 (total 3H, m, –$CH_2C\underline{H}_3$), 2.07—2.33 (total 2H, m, –$C\underline{H}_2CH_3$), 2.98 (59/100 × 1H, d, J=15.5 Hz, –C$\underline{H}$HPh), 3.4—3.8 (59/100 × 1H, dt, J=4.5, 8 Hz, >CHCO), 3.8—4.0 (41/100 × 1H, m, >CHCO), 4.27—4.56 (59/100 × 2H, m, >C$\underline{H}$SPh, –CH$\underline{H}$Ph, 41/100 × 1H, m, –C$\underline{H}$HPh), 4.83 (41/100 × 1H, d, J=14.5 Hz, –CH$\underline{H}$Ph), 6.5—6.8, 7.1—7.4 (total 5H, m, $CH_2\underline{Ph}$), 7.5—7.8 (5H, m, S(O)Ph). MS m/z: 313 (M^+), 312 (M^+ – 1), 188 (M^+ – S(O)Ph).

***N*-(1,1-Diphenymethyl)-3-ethyl-4-(phenylsufinyl)azetidin-2-one (18h)** This (33.9 mg, 78%, *cis*:*trans*=44:56) was obtained from **17h** (41.9 mg, 0.112 mmol), *m*-CPBA (80%, 24.2 mmol, 0.112 mmol) in CH_2Cl_2 (1 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1760, 1045. ^{1}H-NMR ($CDCl_3$) δ: 0.41, 0.59 (56/100 × 3H, t, J=7 Hz, –$CH_2C\underline{H}_3$), 1.15—1.55 (56/100 × 2H, m, –$C\underline{H}_2CH_3$, 44/100 × 3H, m, –$CH_2C\underline{H}_3$), 1.98—2.51 (44/100 × 2H, br, –$C\underline{H}_2CH_3$), 3.04, 3.68 (56/100 × 1H, td, J=7, 2Hz, >CHCO), 3.35—3.58 (44/100 × 1H, m, >CHCO), 3.71, 4.12 (56/100 × 1H, d, J=2 Hz, >C$\underline{H}$SPh), 4.41, 4.47 (44/100 × 1H, d, J=5 Hz, >C$\underline{H}$SPh), 4.60, 5.27 (44/100 × 1H, s, C$\underline{H}$$Ph_2$), 5.70, 6.12 (56/100 × 1H, s, C$\underline{H}$$Ph_2$), 6.98—7.53 (15H, m, ArH). Exact MS Calcd for $C_{24}H_{23}NO_2S$ – SPh: 264.1388. Found: 264.1399.

General Procedure for the Reaction of 4-Phenylsulfinylazetidin-2-ones (18a—c, f—h) with the Ketene Silyl Acetal (16) The ketene silyl acetal (**16**, 2—4 mmol) was added to a stirred solution of 4-phenylsulfinylazetidin-2-one (**18**, 1 mmol) and ZnI_2 (0.05—0.1 mmol) in dry CH_3CN (10 ml) under nitrogen. The mixture was stirred at the temperature and for the period indicated in Table III, then partitioned between CH_2Cl_2 (20 ml) and saturated aqueous $NaHCO_3$ (20 ml). The aqueous layer was extracted with CH_2Cl_2 (20 ml × 4). The combined extract was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography or preparative TLC on silica gel with hexane–AcOEt to give the ester.

***N*-(*tert*-Butyldimethylsilyl)-4-(methoxycarbonylmethyl)azetidin-2-one (19a)**: i) This (7.3 mg, 86%) was obtained from **18a** (10.3 mg, 0.033 mmol), **16** (23.3 mg, 0.124 mmol), and ZnI_2 (1.1 mg, 0.0033 mmol) in CH_3CN (0.5 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.22, 0.25 (total 6H, each s, Me_2Si), 0.96 (9H, s, *tert*-BuSi), 2.49 (1H, dd, J=9.8, 15.9 Hz, –C$\underline{H}$HCO_2Me), 2.77 (1H, dd, J=2.5, 15.9 Hz, –C$\underline{H}$HCO), 2.87 (1H, dd, J=3.7, 15.9 Hz, –CH$\underline{H}$$CO_2$Me), 3.30 (1H, dd, J=5.5, 15.9 Hz, –CH$\underline{H}$CO), 3.70 (3H, s, OMe), 3.89 (1H, m, >C$\underline{H}$$CH_2$). MS m/z: 243 (M^+ – Me), 200 (M^+ – *tert*-Bu). Exact MS Calcd for $C_{12}H_{23}NO_3Si$ – *tert*-Bu: 200.0740. Found: 200.0737. ii) This (7.2 mg, 50%) was obtained from **18a** (17.4 mg, 0.0563 mmol), **16** (21.2 mg, 0.113 mmol), and ZnI_2 (1.8 mg, 0.0056 mmol) in CH_2Cl_2 (1 ml) as a colorless oil. iii) This (7.3 mg, 60%) was obtained from **18a** (14.7 mg, 0.0563 mmol), and **16** (17.9 mg, 0.0952 mmol), and ZnI_2 (1.5 mg, 0.0048 mmol) in THF (1 ml) as a colorless oil. iv) This (14.3 mg, 68%) was obtained from **18i** (16.0 mg, 0.0821 mmol), **16** (38.6 mg, 0.205 mmol), and ZnI_2 (2.6 mg, 0.00821 mmol) in CH_3CN (1 ml) as a colorless oil.

***N*-Benzyl-4-(methoxycarbonylmethyl)azetidin-2-one (19b)** This (6.7 mg, 52%) was obtained from **18b** (16.0 mg, 0.056 mmol), **16** (34 mg, 0.18 mmol), and ZnI_2 (5 mg, 0.0157 mmol) in CH_3CN (1 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1740, 1440, 1400. ^{1}H-NMR ($CDCl_3$) δ: 2.50 (2H, dd, J=2, 6.5 Hz, –$C\underline{H}_2CO_2$Me), 2.67 (1H, dd, J=2, 14.5 Hz, –C$\underline{H}$HCO), 3.11 (1H, dd, J=5, 14.5 Hz, –CH$\underline{H}$CO), 3.56 (3H, s, –OMe), 3.76—4.02 (1H, m, >C$\underline{H}$$CH_2CO_2$), 4.20, 4.45 (2H, AB-q, J=15 Hz, –$C\underline{H}_2$Ph), 7.22 (5H, s, ArH). Exact MS Calcd for $C_{13}H_{15}NO_3$: 233.1047. Found: 233.1047.

***N*-(1,1-Diphenymethyl)-4-(methoxycarbonylmethyl)azetidin-2-one (19c)** This (9.1 mg, 89%) was obtained from **18c** (12.0 mg, 0.0294 mmol), **16** (12.5 mg, 0.0664 mmol), and ZnI_2 (1.1 mg, 0.00332 mmol) in CH_3CN (0.5 ml) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1745. ^{1}H-NMR ($CDCl_3$) δ: 2.35 (1H, d, J=1.2 Hz, –C$\underline{H}$HCO_2Me), 2.44 (1H, s, –CH$\underline{H}$$CO_2$Me), 2.74 (1H, dd, J=2.4, 14.2 Hz, –C$\underline{H}$HCO), 3.22 (1H, dd, J=5.0, 14.2 Hz, –CH$\underline{H}$CO), 3.60 (3H, s, –OMe), 4.00 (1H, m, >C$\underline{H}$$CH_2CO_2$), 5.95 (1H,

s, CHPh$_2$), 7.27 (10H, m, ArH). Exact MS Calcd for $C_{19}H_{19}NO_3$: 309.1363. Found: 309.1355.

***N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(methoxycarbonylmethyl)azetidin-2-one (19f)** i) This (9.1 mg, 79%) was obtained from **18f** (*cis*:*trans* = 63:37, 13.6 mg, 0.0404 mmol), **16** (15.2 mg, 0.0808 mmol), and ZnI_2 (1.3 mg, 0.0040 mmol) in CH_3CN (1 ml) as a colorless oil; ^{1}H-NMR showed *trans*:*cis* = 94:6. The pure isomers (*trans*-**19f** and *cis*-**19f**) were isolated by column chromatography for characterization. ii) This (12.9 mg, 73%) was obtained from *cis*-**18f** (21.0 mg, 0.0623 mmol), **16** (23.4 mg, 0.125 mmol), and ZnI_2 (2.0 mg, 0.0062 mmol) in CH_3CN (1 ml) as a colorless oil; ^{1}H-NMR showed *trans*:*cis* = 95:5. iii) This (13.2 mg, 77%) was obtained from *trans*-**18f** (20.2 mg, 0.0599 mmol), **16** (22.6 mg, 0.120 mmol), and ZnI_2 (1.9 mg, 0.0060 mmol) in CH_3CN (1 ml) as a colorless oil; ^{1}H-NMR showed *trans*:*cis* = 95:5.

(3*R**,4*R**)-*N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(methoxycarbonylmethyl)azetidin-2-one (*trans*-**19f**): IR ν_{max} ($CHCl_3$) cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.20, 0.25 (total 6H, each s, Me$_2$Si), 0.96 (9H, s, *tert*-BuSi), 1.00 (3H, t, J=7.2 Hz, –CH$_2$CH$_3$), 1.75 (2H, m, –CH$_2$CH$_3$), 2.50 (1H, dd, J=9.8, 15.5 Hz, –CHHCO$_2$Me), 2.84 (1H, dd, J=4.3, 15.5 Hz, –CHHCO$_2$Me), 2.88 (1H, ddd, J=2.4, 6.0, 7.0 Hz, >CHCO), 3.59 (1H, ddd, J=2.4, 4.3, 9.8 Hz, >CHCH$_2$CO$_2$), 3.70 (3H, s, OMe). Exact MS Calcd for $C_{14}H_{27}NO_3Si$ – *tert*-Bu: 228.1053. Found: 228.1041.

(3*R**,4*S**)-*N*-(*tert*-Butyldimethylsilyl)-3-ethyl-4-(methoxycarbonylmethyl)azetidin-2-one (*cis*-**19f**): IR ν_{max} ($CHCl_3$) cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.20, 0.23 (total 6H, each s, Me$_2$Si), 0.95 (9H, s, *tert*-BuSi), 1.06 (3H, t, J=7.2 Hz, –CH$_2$CH$_3$), 1.65—1.81 (2H, m, –CH$_2$CH$_3$), 2.58 (1H, dd, J=9.8, 16.5 Hz, –CHHCO$_2$Me), 2.70 (1H, dd, J=5.5, 16.5 Hz, –CHHCO$_2$Me), 3.28 (1H, ddd, J=5.5, 5.9, 10.5 Hz, >CHCO), 3.70 (3H, s, OMe), 4.09 (1H, ddd, J=4.3, 5.5, 9.8 Hz, >CHCH$_2$CO$_2$). MS m/z: 228 (M$^+$ – *tert*-Bu).

***N*-Benzyl-3-ethyl-4-(methoxycarbonylmethyl)azetidin-2-one (19g)** This (4.8 mg, 84%) was obtained from **18g** (6.9 mg, 0.0220 mmol), **16** (8.3 mg, 0.0441 mmol), and ZnI_2 (0.7 mg, 0.0022 mmol) in CH_3CN (0.5 ml) as a colorless oil; ^{1}H-NMR showed *trans*:*cis* = 89:11. IR ν_{max} ($CHCl_3$) cm^{-1}: 1725, 1440, 1400. ^{1}H-NMR ($CDCl_3$) δ: 0.98 (89/100 × 3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.08 (11/100 × 3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.65—1.84 (2H, m, –CH$_2$CH$_3$), 2.47 (11/100 × 2H, d, J=6.7 Hz, –CH$_2$CO$_2$Me), 2.48 (89/100 × 1H, dd, J=15.9, 7.3 Hz, –CHHCO$_2$Me), 2.58 (89/100 × 1H, dd, J=15.9, 6.1 Hz, –CHHCO$_2$Me), 2.85 (89/100 × 1H, ddd, J=1.8, 7.0, 8.0 Hz, >CHCH$_2$CO$_2$), 3.19 (11/100 × 1H, ddd, J=5.5, 6.9, 9.0 Hz, >CHCH$_2$CO$_2$), 3.55 (89/100 × 1H, ddd, J=1.8, 6.1, 7.3 Hz, >CHCO), 3.58 (11/100 × 3H, s, –OMe), 3.61 (89/100 × 3H, s, –OMe), 4.03 (11/100 × 1H, dt, J=5.5, 6.7 Hz, >CHCO), 4.17, 4.55 (89/100 × 2H, each d, J=15.3 Hz, –CH$_2$Ph), 4.25, 4.55 (11/100 × 2H, each d, J=15.3 Hz, –CH$_2$Ph), 7.24—7.35 (10H, m, ArH). Exact MS Calcd for $C_{15}H_{19}NO_3$: 261.1362. Found: 261.1362.

***N*-(1,1-Diphenymethyl)-3-ethyl-4-(methoxycarbonylmethyl)azetidin-2-one (19h)** This (21.0 mg, 80%) was obtained from **18h** (30.3 mg, 0.779 mmol), **16** (29.3 mg, 0.156 mmol), and ZnI_2 (2.4 mg, 0.0078 mmol) in CH_3CN (0.8 ml) as a colorless oil; ^{1}H-NMR showed *trans*:*cis* = 91:9. IR ν_{max} ($CHCl_3$) cm^{-1}: 1735, 1495, 1440, 1380. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (91/100 × 3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.09 (9/100 × 3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.75 (2H, m, –CH$_2$CH$_3$), 2.345 (9/100 × 1H, dd, J=5.5, 17 Hz, –CHHCO$_2$Me), 2.38 (91/100 × 1H, dd, J=8.5, 15.9 Hz, –CHHCO$_2$Me), 2.44 (91/100 × 1H, dd, J=5.5, 15.9 Hz, –CHHCO$_2$Me), 2.46 (9/100 × 1H, dd, J=8, 17 Hz, –CHHCO$_2$Me), 2.85 (91/100 × 1H, dt, J=1.8, 7.3 Hz, >CHCO), 3.20 (9/100 × 1H, ddd, J=5.5, 6.1, 9.8 Hz, >CHCO), 3.57 (9/100 × 3H, s, –OMe), 3.59 (91/100 × 3H, s, –OMe), 3.66 (91/100 × 1H, ddd, J=1.8, 5.5, 8.5 Hz, >CHCH$_2$CO), 4.15 (9/100 × 1H, dt, J=9.8, 5.5 Hz, >CHCH$_2$CO), 5.92 (9/100 × 1H, s, –CHPh$_2$), 5.95 (91/100 × 1H, s, CHPh$_2$), 7.24—7.40 (10H, m, ArH). Exact MS Calcd for $C_{21}H_{23}NO_3$: 337.1676. Found: 337.1671.

(3*R,4*R**)-4-(Benzyloxycarbonylmethyl)-*N*-(*tert*-butyldimethylsilyl)-3-ethylazetidin-2-one (20)** Titanium tetraisopropoxide (11.1 mg, 0.0389 mmol) was added to a stirred solution of *trans*-**19f** (11.1 mg, 0.0389 mmol) in benzyl alcohol (0.5 ml) at room temperature. The mixture was stirred at 80 °C for 2 h and then 1 N hydrochloric acid was added. The aqueous layer was extracted with ether (20 ml × 5) and the combined organic layer was washed with saturated aqueous $NaHCO_3$ (20 ml) and brine (30 ml), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel with hexane–AcOEt (5:1) to give **21** (13.8 mg, 98%) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.18, 0.22 (total 6H, each s, Me$_2$Si), 0.91 (9H, s, *tert*-BuSi), 1.00 (3H, t, J=8 Hz, –CH$_2$CH$_3$), 1.51—1.84 (2H, m, –CH$_2$CH$_3$), 2.47 (1H, dd, J=9.5, 15 Hz, –CHHCO$_2$Me), 2.76—2.98 (2H, m, –CHHCO$_2$Me, >CHCO), 3.58 (1H, ddd, J=2.5, 4, 9.5 Hz, >CHCH$_2$CO$_2$), 5.09 (2H, s, –CH$_2$Ph), 7.33 (5H, s, Ph). Exact MS Calcd for $C_{16}H_{22}NO_3Si$ – *tert*-Bu: 304.1369. Found: 304.1371.

(3*R,4*R**)-4-(Benzyloxycarbonylmethyl)-3-ethylazetidin-2-one (21)** A solution of $Bu_4NF \cdot 3H_2O$ (14.8 mg, 0.0470 mmol) and AcOH (4.8 mg, 0.0754 mmol) in THF (0.5 ml) was added dropwise to a stirred solution of **20** (13.6 mg, 0.0377 mmol) in THF (1 ml) at 0 °C. The mixture was stirred at 0 °C for 30 min, diluted with CH_2Cl_2 (50 ml), washed with water (20 ml) and brine (20 ml), dried over $MgSO_4$, and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with CH_2Cl_2–MeOH (20:1) to give **21** (7.9 mg, 8.5%) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 3420, 1755. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, t, J=7.5, –CH$_2$CH$_3$), 1.76 (2H, m, –CH$_2$CH$_3$), 2.64—2.82 (total 3H, m, –CH$_2$CO$_2$, >CHCO), 3.67 (1H, m, –CHCH$_2$CO$_2$), 5.11 (2H, s, –CH$_2$Ph), 5.96—6.11 (1H, br, NH), 7.31 (5H, s, Ph). Exact MS Calcd for $C_{14}H_{17}NO_3$: 247.1209. Fround: 247.1224.

(3*R,4*R**)-4-Carboxymethyl-3-ethylazetidin-2-one (22)** A 10% Pd–C catalyst (4.2 mg) was added to a stirred solution of **21** (7.4 mg, 0.030 mmol) in ethanol (1 ml) at room temperature. The apparatus was filled with hydrogen and the mixture was stirred at room temperature for 10 min. Pd–C was removed by filtration and the solvent was removed *in vacuo* to give the acid, which was purified by recrystallization to give **22** (3.3 mg, 70%) as colorless crystals, mp 105—108 °C (CH_2Cl_2–C_6H_6) (lit.[12] 105—108 °C (CH_2Cl_2–C_6H_6)). IR ν_{max} ($CHCl_3$) cm^{-1}: 3420, 2280—3600, 1730. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.73, 1.82 (each 1H, each quint, t, J=7.3, 14.7 Hz –CH$_2$CH$_3$), 2.61 (1H, dd, J=9.8, 16.5 Hz, –CHHCO$_2$H), 2.79 (1H, dd, J=4.3, 16.5 Hz, –CHHCO$_2$H), 2.80—2.83 (1H, m, >CHCO), 3.65 (1H ddd, J=1.8, 4.3, 9.8 Hz, >CHCH$_2$CO$_2$H), 6.53 (1H, br, NH). MS m/z: 158 (MH*).

(3*R,4*R**)-4-(3-Benzyloxycarbonyl-3-diazo-2-oxopropyl)-*N*-(*tert*-butyldimethylsilyl)-3-ethylazetidin-2-one (24)** The silyl enol ether (**23**, 103.8 mg, 0.3125 mmol) was added to a stirred solution of 4-phenylsulfinylazetidin-2-one (**18f**, 42.0 mg, 0.125 mmol) and ZnI_2 (4.0 mg, 0.0125 mmol) in dry CH_3CN (1 ml) under nitrogen. The mixture was stirred at room temperature for 15 min, then partitioned between CH_2Cl_2 (20 ml) and saturated aqueous $NaHCO_3$ (20 ml). The aqueous layer was extracted with CH_2Cl_2 (20 ml × 4). The combined extract was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to preparative TLC on silica gel with hexane–AcOEt to give the ester **24** (21.6 mg, 40%) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 2150, 1275, 1645. ^{1}H-NMR ($CDCl_3$) δ: 0.24, 0.25 (total 6H, each s, Me$_2$Si), 0.97 (9H, s, *tert*-BuSi), 0.99 (3H, t, J=7.3 Hz, –CH$_2$CH$_3$), 1.77 (2H, m, –CH$_2$CH$_3$), 2.81 (1H, dt, J=1.8, 6.7 Hz, 3-H), 3.02 (1H, dd, J=9.8, 17.1 Hz, –CHHC=O), 3.45 (1H, dd, J=3.7, 17.1 Hz, –CHHC=O), 3.65 (1H, m, 4-H), 5.28, 5.29 (total 2H, each s, –CH$_2$Ph), 7.38 (5H, m, Ph). Exact MS Calcd for $C_{22}H_{31}N_3O_4Si$ – *tert*-Bu: 372.1378. Found: 372.1378.

(3*R,4*R**)-4-(3-Benzyloxycarbonyl-3-diazo-2-oxopropyl)-3-ethylazetidin-2-one (25)**[13,14] A solution of $Bu_4NF \cdot 3H_2O$ (18.4 mg, 0.0585 mmol) and AcOH (7.0 mg, 0.117 mmol) in THF (1 ml) was added dropwise to a stirred solution of **24** (25.1 mg, 0.0585 mmol) in THF (0.5 ml) at 0 °C. The mixture was stirred at the same temperature for 30 min, diluted with CH_2Cl_2 (50 ml), washed with water (20 ml) and brine (20 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with hexane–AcOEt (3:2) to give **25** (17.0 mg, 93%) as a colorless oil. IR ν_{max} ($CHCl_3$) cm^{-1}: 3460, 2180, 1760, 1720, 1645. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz, –CH$_2$CH$_3$), 1.73 (2H, m, –CH$_2$CH$_3$), 2.75 (1H, m, 3-H), 3.00 (1H, dd, J=8, 18 Hz, –CHHC=O), 3.41 (1H, dd, J=4, 18 Hz, –CHHC=O), 3.68 (1H, m, 4-H), 5.26 (2H, s, –CH$_2$Ph), 6.08 (1H, br s, NH), 7.41 (5H, s, Ph).

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September 1991 Chem. Pharm. Bull. 39(9) 2233—2240 (1991) 2233

Tannins and Related Polyphenols of Melastomataceous Plants. I. Hydrolyzable Tannins from *Tibouchina semidecandra* COGN.

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Four new hydrolyzable tannins, methylvescalagin and nobotanins, A, D and F, along with eleven known polyphenolics, have been isolated from the leaves of *Tibouchina semidecandra* COGN. Based on spectral and chemical evidence, nobotanin D was characterized as 1,6-di-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (16), and the structures of nobotanins A (17) and F (18) were established as dimeric hydrolyzable tannins possessing a valoneoyl group as a linking unit between the monomers.

Keywords *Tibouchina semidecandra*; Melastomataceae; tannin; dimeric ellagitannin; methylvescalagin; nobotanin A; nobotanin D; nobotanin F

Melastomataceous plants are widely distributed in tropical and subtropical areas such as South America, Southeast Asia and the southern part of China. Some of them have been used as remedies for diarrhoea, dysentery, leucorrhea, and various skin diseases, and also as astringents and hemostatics, in Indonesia, Malaysia and China.[1] These activities are attributable to tannins, and the occurrence of ellagic acid and its alkylated derivatives in a variety of plants of this family has been reported,[2] implying the presence of ellagitannins in these plants. However, no phytochemical study on the tannins of melastomataceous plants has been reported.

We have now found by means of a chromatographic survey that *Tibouchina semidecandra* COGN., which is native to Brazil, is rich in tannins, particularly in oligomeric hydrolyzable tannins. As chemical constituents of this plant, several flavonoids (quercetin, myricetin, tibouchinin, leucodelphinidin and leucocyanidin) have been reported.[3] Ellagic acid was reported to be present in the acid hydrolyzates of the methanol extract of the leaves.[4] The present paper deals with the isolation and characterization of fifteen polyphenols, including four new hydrolyzable tannins, from the leaf extract of this plant.

The aqueous acetone homogenate of the fresh leaves was concentrated and extracted successively with diethyl ether, ethyl acetate and *n*-butanol. The ethyl acetate extract was chromatographed over Sephadex LH-20, to give nobotanins A, D and F, and eight known compounds. Among the known compounds, two were flavonoid glycosides which were characterized as quercetin-3-*O*-(6″-galloylgalactoside)[5] and quercetin-3-*O*-α-L-arabinofuranoside,[6] based on their physicochemical data. The other six were hydrolyzable tannins, and were identified as pedunculagin (**1**),[7] casuarictin (**2**),[7] praecoxins A (**3**)[8] and B (**4**),[9] 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose(**5**),[10] and casuarinin (**10**).[7] An analogous chromatographic separation of the *n*-butanol extract afforded 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**7**)[11] and three *C*-glucosidic tannins (**13**—**15**).

The ethyl acetate extract, from the crude extract with aqueous acetone of the fresh stems of the same plant, was similarly chromatographed over Sephadex LH-20 to yield 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose (**9**), in addition to **1**, **4**, **5**, **10**, and nobotanins A (**17**) and F (**18**).[12]

Among the three *C*-glucosidic tannins isolated from the *n*-butanol extract of the leaves, two were identified as castalagin (**13**)[7b,13] and vescalagin (**14**).[7b,14] The third

1 : R=H, R'=OH
1a: R=Me, R'=(β)-OMe
2 : R=H, R'=(β)-OG

3

4 : R=H, R'=OH, R"=G
5 : R=R"=H, R'=OH
5a: R=Me, R'=OMe, R"=H
6 : R=H, R'=(β)-OG, R"=G

7 : R=OH
8 : R=(β)-OG

9

G= OC–(3,4,5-trihydroxyphenyl)

Chart 1

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tannin (**15**), $[\alpha]_D$ $-75°$ (MeOH), was isolated as an off-white amorphous powder. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **15** exhibited three aromatic 1H singlets at δ 6.76, 6.75 and 6.60, and a methoxyl proton signal at δ 3.60. The sugar proton signals showed the characteristic pattern of an open-chain glucose core, as found in **13** and **14**. The coupling constant (J=2 Hz) of the H-1 signal (δ 4.66) in **15** is the same as that of vescalagin (**14**), although it is shifted upfield by 0.25 ppm relative to that of the latter. A downfield shift of the H-2 signal (δ 5.33) from that (δ 5.28) of **14** suggests that **15** is 1-*O*-methylvescalagin. This assumption was substantiated by the fast-atom bombardment mass spectrum (FAB-MS) [m/z 971 $(M+Na)^+$], and also by production of **15** upon treatment of **14** with methanol in the presence of a catalytic amount of trifluoroacetic acid at 37 °C. On the other hand, **15** was gradually converted into **14** when an aqueous solution of **15** was kept at 37 °C.

The rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY) of **15** showed nuclear Overhauser effect's (NOE's) between the H-1 and H-3 signals, and also between H-2 and *O*-methyl signals, indicating β-configuration of the methoxyl group at C-1. This configuration is consistent with the recent revision[7b] of the C-1 configurations of **14** and its analog, stachyurin (**11**),[7b] which were previously assigned to be α based on their coupling constant (J=2 Hz) of the H-1 signal.[7a] It is noticeable that the chemical transformation between **15** and **14** mentioned above proceeds with retention of the C-1 configuration, as judged from the identity of the coupling constant (J=2 Hz) before and after the reaction. In contrast to the high reactivities of **14**, **15** and **11** toward mild solvolysis at C-1 of the glucose core, their C-1 epimers, **13** and casuarinin (**10**),[7,15] were unaffected under the same conditions, as summarized in Table I. These differences may be interpreted in terms of lesser steric hindrance at the β-site of C-1 and/or stabilization of the C-1 α-hydroxyl group by hydrogen bonding with a nearby hydroxyl group of the hexahydroxydiphenoyl (HHDP) part at C-1/O-2/O-3. In fact, the steric proximity between the C-1 α-site and the aromatic ring of the HHDP group at O-4/O-6 was shown by the NOE's among H-1, H-2 and the HHDP proton signal at δ 6.75 in ROESY of **15** (Fig. 1). The facile transformation of **14** into **15** may indicate that **15** is an artefact produced during the chromatographic separation.

Nobotanin D (**16**), $[\alpha]_D$ $-68°$ (MeOH), was obtained as a light brown amorphous powder, and showed the positive coloration characteristic of ellagitannins with the $NaNO_2$–AcOH reagent.[16] The ^{1}H-NMR spectrum of **16** indicated the presence of two galloyl groups [δ 7.12 and 7.11 (each 2H, s)] and an HHDP group [δ 6.70 and 6.42 (each 1H, s)]. The sugar proton signals are typical of $^4C_1$ glucopyranose, and resonate at the lower field than 4.40 ppm, except for H-4 (δ 3.98, t, J=10 Hz) and H-5 (δ 4.07, m, H-5). The acyl groups in **16** thus should be located at O-1, O-2, O-3 and O-6. The β-orientation of the acyloxy group at the anomeric center of **16** is evident from the large coupling constant (J=8 Hz) of the H-1 signal (δ 6.17). Since

10 : R=H, R'=OH
11 : R=OH, R'=H
12 : R=OMe, R'=H

13 : R=H, R'=OH
14 : R=OH, R'=H
15 : R=OMe, R'=H

Chart 2

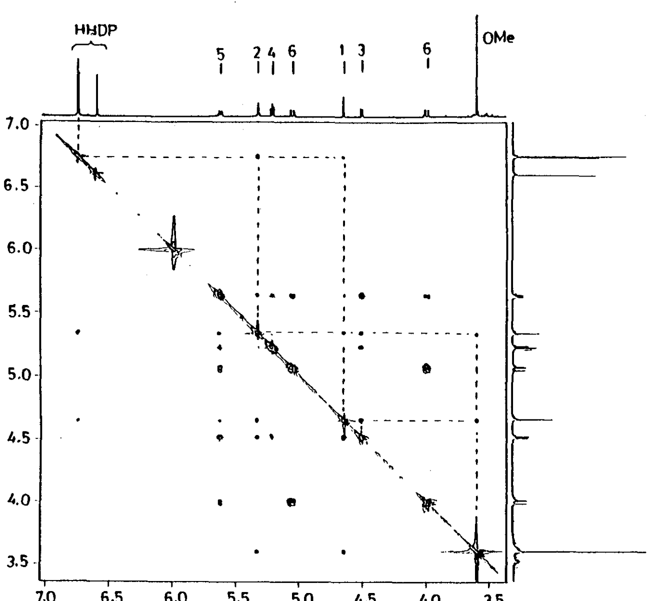

Fig. 1. ROESY Spectrum of **15** (500 MHz, Acetone-d_6)

TABLE I. Solvolysis[a] of *C*-Glucosidic Tannins

	Solvent	Product	Yield (%)[b] 6 h	25 h	50 h	75 h	100 h
14	MeOH/CF_3COOH	**15**	100				
	MeOH	**15**	10	42	70	85	95
15	H_2O	**14**	5	17	30	40	50
13	MeOH/CF_3COOH	n[c]					
11	MeOH/CF_3COOH	**12**	100				
10	MeOH/CF_3COOH	n[c]					

a) Reaction was carried out with a 0.1% solution at 37 °C. *b*) Yield was determined by reversed-phase HPLC [YMC-pack A312, 0.1 M phosphate buffer–EtOH 100:5]. *c*) n, no reaction.

16

Chart 3

the HHDP group in the ellagitannins possessing the $^4C_1$ glucopyranose core can be located only at O-4/O-6 or O-2/O-3,[17] nobotanin D is assumed to be 1,6-di-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (**16**). This structure (**16**) was confirmed by treatment of nobotanin D with tannase,[18] yielding 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**5**) and 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**7**).

Nobotanins A (**17**) and F (**18**) are ellagitannin dimers as evidenced by the coloration with the $NaNO_2$–AcOH reagent, which was similar to that of **16**, and by their retention times on high-performance liquid chromatography (HPLC) (normal phase),[19] which are longer than those of the monomeric hydrolyzable tannins such as **2**, **3** and **16**.

Nobotanin F (**18**), $[\alpha]_D$ +60° (MeOH), showed an $(M+Na)^+$ peak at *m/z* 1895 in FAB-MS. Acid hydrolysis of **18** gave gallic acid (**19**), ellagic acid (**20**), valoneic acid dilactone (**22**), and glucose. The ^{1}H-NMR spectrum of **18** indicated the presence of three galloyl (δ 7.16, 7.15, 7.14, each 2H, s), two HHDP and a valoneoyl groups (δ 7.07, 6.49, 6.46, 6.44, 6.42, 6.41, 6.22, each 1H, s). The *S* configuration of both HHDP and valoneoyl groups was shown by the circular dichroism (CD) spectrum of **18**, which exhibited a strong positive Cotton effect at 231 ($[\theta]$ $+32.3\times10^4$) and 235 nm ($[\theta]$ $+32.6\times10^4$).[20] The $^4C_1$ conformation of two glucose cores was revealed by the coupling pattern of their signals, among which the anomeric proton signals were observed as doublets (J=8.5 Hz) at δ 6.18 and 6.17. The glycosidic linkages at C-1 are accordingly *β*. The methylene proton signals of a glucose core (glucose-I) [δ 4.46 (br d, J=13 Hz) and 4.24 (dd, J=3, 13 Hz)] are similar to those of **5** and **9**, and suggest the presence of a galloyl group or the galloyl part of a valoneoyl group at

Chart 4

TABLE II. ^{1}H-NMR Data[a] for the Glucose Moieties of **2**, **4**, **6**, **17** and **18** (500 MHz, Acetone-d_6+D_2O, J in Hz)

	2	**4**		**6**	**17**		**18**[b]
		α-Anomer	β-Anomer		α-Anomer	β-Anomer	
Glucose-I							
H-1		5.48 d (J=3.5)	5.17 d (J=8)	6.30 d (J=8.5)	5.46 d (J=3.5)	5.16 d (J=8)	6.18 d (J=8.5)
H-2		5.07 dd (J=3.5, 9.5)	4.88 dd (J=8, 9.5)	5.21 t (J=8.5)	5.03 dd (J=3.5, 10)	4.82 dd (J=8, 10)	5.14 dd (J=8.5, 9.3)
H-3		5.62 t (J=9.5)	5.35 t (J=9.5)	5.55 dd (J=8.5, 9)	5.60 t (J=10)	5.15 t (J=10)	5.44 t (J=9.3)
H-4		5.50 t (J=9.5)	5.46 t (J=9.5)	5.59 t (J=9)	5.50 t (J=10)	5.54 t (J=10)	5.58 t (J=9.3)
H-5		4.54 ddd (J=2, 4, 9.5)	4.22 ddd (J=2, 5, 9.5)	4.50 dd (J=5, 9)	4.37 m	3.84 br d (J=10)	4.17 br dd (J=3, 9.3)
H-6		4.49 dd (J=2, 12)	4.49 dd	4.55 d (J=12)	4.39 br d (J=12.5)	4.61 br d (J=12.5)	4.46 br d (J=13)
		4.27 dd (J=4, 12)	4.26 dd (J=5, 12)	4.32 dd (J=5, 12)	4.16 dd (J=4, 12.5)	4.25 dd (J=3, 12.5)	4.24 dd (J=3, 13)
Glucose-II							
H-1′	6.22 d (J=9)				6.14 d (J=8.5)	6.16 d (J=8.5)	6.17 d (J=8.5)
H-2′	5.18 t (J=9)				5.27 t (J=8.5)	5.34 t (J=8.5)	5.19 t (J=8.5)
H-3′	5.45 dd (J=9, 10)				5.39 dd (J=8.5, 10)	5.42 dd (J=8.5, 10)	5.42 dd (J=8.5, 10)
H-4′	5.17 t (J=10)				5.12 t (J=10)		5.10 t (J=10)
H-5′	4.50 dd (J=7, 10)				4.43 br dd (J=6.5, 10)		4.42 br dd (J=6, 10)
H-6′	5.30 dd (J=7, 13)				5.17 dd (J=6.5, 13)	5.22 dd (J=6.5, 13)	5.14 dd (J=6, 13)
	3.88 d (J=13)				3.74 br d (J=13)		3.70 br d (J=13)

a) Assigned by ^{1}H–^{1}H COSY. b) Measured at 400 MHz.

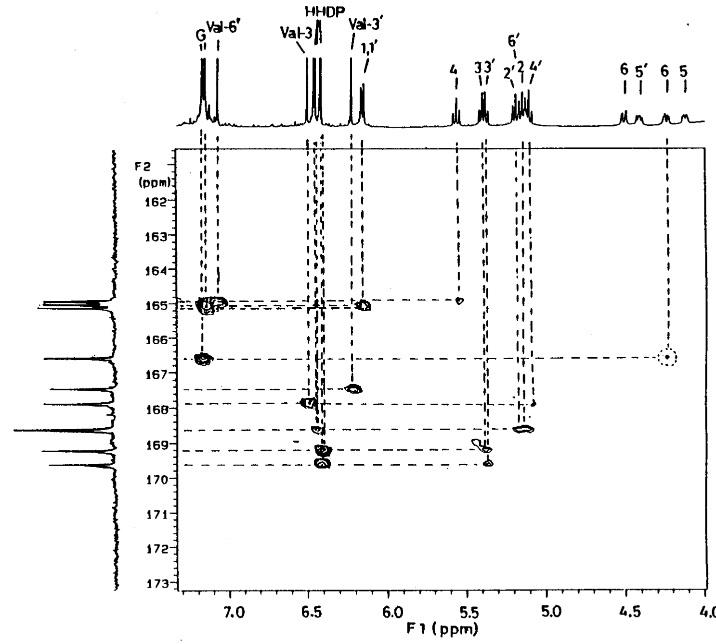

Fig. 2. A Part of the ^{1}H–^{13}C Long-Range Correlation Two-Dimensional NMR Spectrum of Nobotanin F (**18**) (δ_C 160—173)

C-6. The methylene protons in the other glucose core were observed at δ 5.14 (dd, J=6, 13 Hz) and 3.70 (br d, J=13 Hz), in a way similar to those of **2**, **3** and other ellagitannins possessing an HHDP group or the HHDP part of the valoneoyl group at O-4/O-6 of the $^{4}C_1$ glucopyranose core.[17] The chemical shifts (δ 92.0 and 92.1) of the anomeric carbon signals are in the diagnostic range (δ 92—94)[21] for the locations of a β-oriented galloyloxy group at C-1 and an HHDP group (or HHDP moiety) at O-2/O-3, of each $^{4}C_1$ glucopyranose core in **18**. These locations are supported

Table III. ^{13}C-NMR Data for the Glucose Moieties of **2**, **4**, **6**, **17** and **18** (126 MHz, Acetone-d_6)

	2	**4**		**6**	**17**[a]		**18**[a]
		α-Anomer	β-Anomer		α-Anomer	β-Anomer	
Glucose-I							
C-1		91.3	94.9	91.9	91.4	94.9	92.0
C-2		75.1	77.7	75.3	75.2	78.0	75.1
C-3		75.4	77.6	77.4	75.6	77.7	77.4
C-4		68.7	68.5	67.8	68.7	68.3	67.7
C-5		68.4	73.1	73.9	68.2	72.8	73.9
C-6		63.1	63.2	62.7	63.2	63.2	62.5
Glucose-II							
C-1′	92.4				92.4	92.4	92.1
C-2′	76.0				75.9	75.9	75.6
C-3′	77.3				77.4	77.4	77.0
C-4′	69.3				69.2	69.4	69.1
C-5′	73.5				73.4	73.4	73.4
C-6′	63.1				63.2	63.2	63.0

a) Measured at 100 MHz.

by the glucose carbon signals in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **18**, which are in good agreement with those of 1,4,6-tri-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (pterocarinin C, **6**)[22] and casuarictin (**2**), as shown in Table III.

The locations of galloyl groups at O-1, O-1′ and O-6 on the glucose cores in **18** were confirmed by the ^{1}H–^{13}C long-range correlation spectroscopy (COSY) as follows. The ester carbonyl carbon resonances (δ 165.0, 165.1, 166.6), which correlated with the galloyl proton signals through three-bond coupling, also exhibit cross peaks with the H-1, H-1′ and H-6 signals as shown in Fig. 2. The 1H singlet at δ 7.07, which resonates at the lowest region among the aromatic 1H singlets, and is thus assigned to H-6″ of the valoneoyl group, was also shown to correlate with the H-4 signal through an ester carbonyl carbon signal at 164.9. Therefore, the galloyl part of the valoneoyl group in **18** should be at O-4 of the glucose core-I.

The structure (**18**) of nobotanin F, thus proposed, was chemically confirmed as follows.

Methylation of **18** with dimethyl sulfate yielded methyl tri-*O*-methylgallate (**19a**), dimethyl hexamethoxydiphenate (**21**), trimethyl octa-*O*-methylvaloneate (**23**) and an octadecamethyl derivative (**24a**), in addition to a permethylated derivative (**18a**).[23] The product (**24a**) was characterized as the methylated derivative of rugosin C (**24**),[24] based on the electron impact-mass spectrum (EI-MS) data [m/z 1356 (M^{+})] and the ^{1}H-NMR spectrum, which exhibits five 1H singlets due to an HHDP group and a valoneoyl group, and a 2H singlet attributable to a galloyl group. On the other hand, partial hydrolysis of **18** in boiling water gave six hydrolyzates, among which three were identified as gallic acid (**19**), 2,3-*O*-(*S*)-hexahydroxydiphenoylglucose (**7**) and isostrictinin (**8**).[25] The structures of the other three hydrolyzates are represented by the formulas **25**, **26** and **27**, as follows. The ^{1}H-NMR spectrum of the hydrolyzate (**25**) indicates the presence of two galloyl groups (δ 6.70 and 7.10), an HHDP group (δ 6.39 and 6.43) and a fully acylated $^{4}C_{1}$ glucose core in the molecule. Three 1H singlets in the low field region (δ 7.16, 7.19 and 7.54) were attributable to the protons of a dilactonized valoneoyl group. The presence of this group was also supported by the ultraviolet (UV) absorption at 363 nm.[26] The hydrolyzate (**26**) was shown to be a degalloyl derivative of **25**, based on the aromatic proton signals due to a galloyl, an HHDP and a dilactonized valoneoyl group, in the ^{1}H-NMR spectrum. Each signal of **26** is duplicated, and this indicates that the galloyl group at C-1 of **25** is lacking in **26**, which exists as an equilibrium mixture of α- and β-anomer (see Experimental). This structural correlation was substantiated by partial hydrolysis of **25** with tannase, yielding **26**. Similarly, **27** was shown to be a monomer related to **25**, lacking an HHDP group at O-2/O-3 of the latter, by the ^{1}H-NMR spectrum which exhibits two 2H singlets (δ 6.88 and 7.11) and three 1H singlets (δ 7.15, 7.24 and 7.54) due to two galloyl groups and a dilactonized valoneoyl group, and also remarkable upfield shifts of H-2 and H-3 relative to those of **25**.

Based on the above spectral and chemical evidence, the structure of nobotanin F was established as **18**.

Nobotanin A (**17**), $[\alpha]_D$ +88° (MeOH), was obtained as an off-white amorphous powder. Acid hydrolysis of **17** gave the same products (gallic acid, ellagic acid, valoneic acid dilactone and glucose) as those from **17**. Although the ^{1}H-NMR spectrum of **17** was complicated by duplication of signals induced by anomerization of the glucose core, the presence of two galloyl, two HHDP, and a valoneoyl group, and of two $^{4}C_{1}$ glucopyranose cores was apparent (see Experimental). The existence of **17** as an equilibrium mixture of α- and β-anomer (2:1) is evident from the anomeric proton signals at δ 5.16 (2/3H, d, J=8 Hz) and 5.46 (1/3H, d, J=3.5 Hz), and the paired signal of an acylated anomeric proton at δ 6.16 and 6.14 (each d, J=8.5 Hz, 1H in total). The chemical shifts and the coupling patterns of the glucose signals in the ^{1}H- and ^{13}C-NMR spectra of **17** are in good agreement with the sum of the signals of praecoxin B (**4**)[9] and casuarictin (**2**) (Tables II and III). Nobotanin A is thus considered to be a degalloyl congener of nobotanin F (**18**). This structure is consistent with the [M+Na]$^{+}$ ion peak at m/z 1743 in FAB-MS, which is 152 mass unit (galloyl fragment) lower than that of **18**, and with the positive Cotton effects at 227 nm ([θ] +24.2×10^{4}) and 235 nm ([θ] +19.7×10^{4}) in the CD spectrum, which are similar to those of **18**. Methylation of **17** with dimethyl sulfate and potassium carbonate provided a permethylate (**17a**), and several partially degraded products, among which three were characterized as **1a**, **24a**, and **5a**. Upon partial hydrolysis in hot water, **17** gave isostrictinin (**8**) and **26**. Finally, a selective degalloylation of **18** with tannase gave **17**.

Based on these findings, the structure of nobotanin A was established to be as illustrated by the formula **17**.

Experimental

Optical rotations were determined on a JASCO DIP-4 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a Hitachi R22-FTS (90 MHz for ^{1}H), Bruker AM-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Varian VXR 500 (500 MHz for ^{1}H and 126 MHz for ^{13}C) instrument, and chemical shifts are given in δ (ppm) relative to tetramethylsilane. EI-MS were taken on a Shimadzu LKB-9000 GC-MS instrument and FAB-MS on a VG 70-SE mass spectrometer using 3-nitrobenzyl alcohol containing NaCl as the matrix agent. HPLC was conducted on a Shimadzu LC-6A apparatus. Normal phase HPLC was carried out on a column of Develosil 60-5 (4 mm i.d. × 15 cm) developed with hexane–MeOH–tetrahydrofuran (THF)–HCOOH (55:33:11:1) containing oxalic acid (450 mg/l) or hexane–MeOH–THF–HCOOH (60:45:15:1) containing oxalic acid 599 mg/l, and reversed-phase HPLC on a column of YMC-pack A312 (6 mm i.d. × 15 cm) with 0.01 M KH_2PO_4–0.01

M H_3PO_4–EtOH–EtOAc (42.5 : 425 : 10 : 5). Column chromatography was performed with Sephadex LH-20 (Pharmacia Fine Chemicals) and Toyopearl HW-40W (Tosoh Co.). Thin-layer chromatography (TLC) was carried out with Kieselgel PF_{254} (Merck). Solvents were removed under reduced pressure below 40 °C.

Isolation of Polyphenols The fresh leaves (1.1 kg) of *Tibouchina semidecandra* cultivated in a greenhouse of the Faculty of Pharmaceutical Sciences, Okayama University, were homogenized in 70% aqueous acetone (3 l × 3), and the homogenates were filtered. After evaporation of acetone, the concentrated aqueous solution (300 ml) was extracted successively with Et_2O (300 ml × 5), EtOAc (300 ml × 20), and *n*-BuOH (300 ml × 18) presaturated with water. Yields of the extracts were as follows: Et_2O extract 1.0 g, EtOAc extract 13 g, *n*-BuOH extract 10 g, residue from aqueous layer 42.7 g. A part (7.5 g) of the EtOAc extract was chromatographed on Sephadex LH-20 (2.2 cm i.d. × 43 cm) with EtOH and an EtOH–MeOH system [(8 : 2)→(7 : 3)→(1 : 1)→(3 : 7)], and with 70% aqueous acetone. The eluate with EtOH gave two fractions successively (frs. A and B), and fr. A (114 mg) was further chromatographed over cellulose (Avicel) using water as an eluant to give quercetin-3-*O*-(6″-*O*-galloylgalactoside) (17 mg) and quercetin-3-*O*-α-L-arabinofuranoside (72 mg). Fraction B gave 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**5**) (196 mg). The eluate with EtOH–MeOH (8 : 2) was also divided into three fractions (frs. C—E), and the eluates from EtOH–MeOH (3 : 7) gave three fractions (frs. F—H). Fraction C was rechromatographed over cellulose (H_2O) to give nobotanin D (**16**) (8 mg) and praecoxin B (**4**) (94 mg). Fraction D was also rechromatographed over Toyopearl HW-40 (70% EtOH) to give pedunculagin (**1**) (75 mg), casuarinin (**10**) (126 mg) and casuarictin (**2**) (455 mg). Praecoxin A (**3**) (56 mg) was obtained from fr. E by similar column chromatography over cellulose (H_2O). Fraction F gave nobotanin A (**17**) (260 mg). Fraction H was further chromatographed over Sephadex LH-20 with EtOH–H_2O–acetone (6 : 3 : 1) to give nobotanin F (**18**) (29 mg). A part (14 g) of the residue from the aqueous layer was applied to a column (3.3 cm i.d. × 45 cm) of Amberlite XAD-2, and eluted with H_2O (1 l) and with MeOH (1.5 l) to give the H_2O eluate (9.9 g) and MeOH eluate (3.8 g). The H_2O eluate (8.5 g) was chromatographed over Toyopearl HW-40 (coarse grade) with 50% MeOH to afford 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**7**) (12 mg), castalagin (**13**) (45 mg), vescalagin (**14**) (46 mg) and 1-*O*-methylvescalagin (**15**) (11 mg).

Fresh stems (417 g) of *T. semidecandra* were homogenized in 70% aqueous acetone (1 l × 3) and filtered. The filtrate was treated in a similar way to that described for the leaves, and the EtOAc extract (2.4 g) was chromatographed over Sephadex LH-20. Elution with EtOH containing increasing amounts of MeOH afforded 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**5**) (17 mg), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**9**) (7 mg), praecoxin B (**4**) (17 mg), pedunculagin (**1**) (281 mg), casuarinin (**10**) (29 mg), nobotanin A (**17**) (178 mg) and nobotanin F (**18**) (124 mg).

Quercetin-3-*O*-(6″-*O*-galloylgalactoside) Yellow needles, mp 210 °C (dec.). UV λ_{max} (MeOH) nm (log ε): 265 (4.43), 290 s (4.21), 357 (4.25). ^{1}H-NMR (90 MHz, CCl_4) of the trimethylsilyl ether δ: 7.70 (1H, dd, *J* = 2, 8 Hz, H-6′), 7.30 (1H, d, *J* = 2 Hz, H-2′), 6.79 (1H, d, *J* = 8 Hz, H-5′), 6.42 (1H, d, *J* = 2 Hz, H-8), 6.11 (1H, d, *J* = 2 Hz, H-6), 7.02 [2H, s, galloyl (G)], 5.62 (1H, d, *J* = 8 Hz, galactose H-1), 4.10 (2H, br s, galactose H-6), 3.5—3.9 (4H, galactose H-2, 3, 4, 5).

Quercetin-3-*O*-α-L-arabinofuranoside Yellow needles, mp 206—208 °C, $[\alpha]_D$ −161° (*c* = 0.5, MeOH). UV λ_{max} (MeOH) nm: 208, 256, 265 sh, 301 sh, 355; (MeOH + $AlCl_3$): 212, 275, 305 sh, 437. ^{1}H-NMR (90 MHz, acetone-d_6) δ: 12.51 (1H, s, 5-OH), 7.73 (1H, d, *J* = 2 Hz, H-2′), 7.60 (1H, dd, *J* = 2, 8 Hz, H-6′), 7.00 (1H, d, *J* = 8 Hz, H-5′), 6.51 (1H, d, *J* = 2 Hz, H-8), 6.28 (1H, d, *J* = 2 Hz, H-6), 5.50 [1H, s, arabinose (Ara) H-1], 4.29—3.61 (Ara H-2, H-5). ^{13}C-NMR (22.6 MHz, DMSO-d_6) δ: 156.9 (C-2), 133.4 (C-3), 177.7 (C-4), 161.1 (C-5), 98.7 (C-6), 164.2 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 121.7 (C-1′), 115.5 (C-2′), 145.0 (C-3′), 148.4 (C-4′), 115.5 (C-5′), 121.0 (C-6′), 107.9 (C-1″), 82.1 (C-2″), 76.9 (C-3″), 85.8 (C-4″), 60.7 (C-5″).

Vescalagin (14) A light brown amorphous powder. ^{1}H-NMR (400 MHz, acetone-d_6 + D_2O) δ: 6.84, 6.80, 6.65 (each 1H, s, HHDP), 4.91 (1H, d, *J* = 2 Hz, H-1), 5.28 (1H, dd, *J* = 2, 2.5 Hz, H-2), 4.57 (1H, dd, *J* = 2, 7 Hz, H-3), 5.22 (1H, t, *J* = 7 Hz, H-4), 5.63 (1H, ddd, *J* = 1, 2.5, 7 Hz, H-5), 4.04 (1H, dd, *J* = 1, 13 Hz, H-6), 5.10 (1H, dd, *J* = 3, 13 Hz, H-6).

Methylvescalagin (15) An off-white amorphous powder, $[\alpha]_D$ −75° (*c* = 1.0, 50% aqueous acetone). FAB-MS *m/z*: 971 (M + Na)$^+$. UV λ_{max} (MeOH) nm (log ε): 228 (4.85). ^{1}H-NMR (500 MHz, acetone-d_6) δ: 6.76, 6.75, 6.60 (each 1H, s, HHDP), 4.66 (1H, d, *J* = 2 Hz, H-1), 5.33 (1H, t, *J* = 2 Hz, H-2), 4.51 (1H, dd, *J* = 2, 7 Hz, H-3), 5.22 (1H, t, *J* = 7 Hz, H-4), 5.63 (1H, ddd, *J* = 1, 2.5, 7 Hz, H-5), 4.00 (1H, br d, *J* = 13 Hz, H-6), 5.06 (1H, dd, *J* = 2.5, 13 Hz, H-6), 3.60 (3H, s, OMe).

Solvolysis of *C*-Glucosidic Tannins with MeOH A mixture of *C*-glucosidic tannin (**11** or **14**) (10 mg) and CF_3COOH (0.1 ml) in absolute MeOH (10 ml) was kept standing at 37 °C overnight, and the solvent was evaporated off to give the 1-*O*-methyl derivative (**12** or **15**) quantitatively.

12: An off-white amorphous powder. FAB-MS *m/z*: 973 (M + Na)$^+$. ^{1}H-NMR (500 MHz, acetone-d_6) δ: 7.09 (2H, s, G), 6.87, 6.53, 6.50 (each 1H, s, HHDP), 4.64 (1H, d, *J* = 2 Hz, H-1), 4.98 (2H, diffused s, H-2, H-3), 5.72 (1H, dd, *J* = 2, 8.5 Hz, H-4), 5.32 (1H, dd, *J* = 3.5, 8.5 Hz, H-5), 4.89 (1H, dd, *J* = 3.5, 13.5 Hz, H-6), 4.00 (1H, d, *J* = 13.5 Hz, H-6), 3.41 (3H, s, OMe).

Nobotanin D (16) A light brown amorphous powder, $[\alpha]_D$ −68° (*c* = 0.1, MeOH). *Anal.* Calcd for $C_{34}H_{26}O_{22} \cdot 7H_2O$: C, 44.74; H, 4.42. Found: C, 44.70; H, 4.11. UV λ_{max} (MeOH) nm (log ε): 219 (4.80), 275 (4.46). ^{1}H-NMR (500 MHz, acetone-d_6 + D_2O) δ: 7.12, 7.11 (each 2H, s, G), 6.70, 6.42 (each 1H, s, HHDP), 6.17 (1H, d, *J* = 8 Hz, H-1), 5.24 (1H, t, *J* = 10 Hz, H-3), 5.05 (1H, dd, *J* = 8, 10 Hz, H-2), 4.60 (2H, dd, *J* = 1.5, 12 Hz, H-6), 4.45 (1H, dd, *J* = 5, 12 Hz, H-6), 4.07 (1H, m, H-5), 3.98 (1H, t, *J* = 10 Hz, H-4). ^{13}C-NMR (126 MHz, acetone-d_6 + D_2O) δ: 92.1 (C-1), 79.9, 76.2, 75.3, 67.8 (C-2, 3, 4, 5), 63.6 (C-6), 107.0, 107.5 (HHDP C-3, C-3′), 109.8, 110.1 (G C-2, C-6), 114.5, 114.6 (HHDP C-1, C-1′), 126.1, 126.7 (G C-1), 126.1, 126.7 (HHDP C-2, C-2′), 136.1, 136.2 (HHDP C-5, C-5′), 138.9, 139.8 (G C-4), 144.2, 144.3, 145.0, 146.1 (HHDP C-4, C-6), 145.1, 145.2 (G C-3, C-5), 165.2, 166.8, 168.9, 169.5 (ester carbonyl).

Partial Hydrolysis of Nobotanin D (16) with Tannase A solution of **16** (1 mg) in H_2O (1 ml) was incubated with tannase at 37 °C for 3 h. The reaction mixture was directly analyzed by HPLC (reversed-phase) to detect peaks identical with those of **5**, **7** and **19**.

Nobotanin A (17) An off-white amorphous powder, $[\alpha]_D$ +88° (*c* = 1.0, MeOH). *Anal.* Calcd for $C_{75}H_{52}O_{48} \cdot 8H_2O$: C, 48.29; H, 3.67. Found: C, 48.25; H, 3.80. FAB-MS *m/z*: 1743 [M + Na]$^+$. UV λ_{max} (MeOH) nm (log ε): 219 (5.07), 270 (4.77). CD (MeOH) [θ] (nm): $+24.2 \times 10^4$ (227), $+19.7 \times 10^4$ (235), -7.1×10^4 (262), $+3.4 \times 10^4$ (282), -1.9×10^4 (303). ^{1}H-NMR (500 MHz, acetone-d_6 + D_2O) α- and β-anomer (2 : 1) δ: 7.15, 7.17 (each s, 4/3H), 7.16, 7.18 (each s, 2/3H) (G), 6.23, 6.35, 6.43, 6.44, 6.50, 6.65, 6.95 (each s, 2/3H), 6.29, 6.41, 6.45, 6.46, 6.53, 6.71, 7.00 (each s, 1/3H) [HHDP and valoneoyl (Val)], glucose protons, see Table II.

Nobotanin F (18) An off-white amorphous powder, $[\alpha]_D$ +60° (*c* = 0.5, MeOH). *Anal.* Calcd for $C_{82}H_{56}O_{52} \cdot 8H_2O$: C, 48.82; H, 3.60. Found: C, 49.14; 3.98. FAB-MS (glycerol + NaI) *m/z*: 1895 [M + Na]$^+$; (glycerol + KI) *m/z*: 1911 [M + K]$^+$. UV λ_{max} (MeOH) (nm) (log ε): 219 (5.20), 272 (4.86). CD (MeOH) [θ] (nm): $+32.3 \times 10^4$ (231), $+32.6 \times 10^4$ (235), -10.2×10^4 (261), $+4.5 \times 10^4$ (282), -1.6×10^4 (310). ^{1}H-NMR (400 MHz, acetone-d_6 + D_2O) δ: 7.14, 7.15, 7.16, (each 2H, s, G), 6.22, 6.41, 6.42, 6.44, 6.46, 6.49, 7.07 (each 1H, s, HHDP and Val), glucose protons, see Table II. ^{13}C-NMR (100 MHz, acetone-d_6 + D_2O) δ: 104.8 (Val C-3′), 107.1 (3C), 107.4, 107.5 (HHDP C-3, C-3′, Val C-3), 109.7 (Val C-6″), 110.0 (2C), 110.1 (4C) (G C-2, C-6), 114.2, 114.3, 114.7, 114.8, 114.9, 115.2, 116.9 (HHDP C-1, C-1′, Val C-1, C-1′, C-1″), 119.4 (2C), 120.9 (G C-1), 125.1, 125.5, 125.6, 125.7, 125.9, 126.1 (HHDP C-2, C-2′, Val C-2, C-2′), 136.0, 136.1, 136.2, 136.3, 136.4 (2C), 136.7 (HHDP C-5, C-5′, Val C-5, C-5′, C-2″), 139.0, 139.9, 140.0, 140.4 (2C) (G C-4, Val C-3″, C-4″), 143.5 (Val C-5″), 144.2, 144.3 (2C), 144.4, 144.6, 145.1 (6C), 145.4 (HHDP C-4, C-4′, C-6, C-6′, Val C-4, C-6, C-6′), 145.8 (2C), 146.1 (4C) (G C-3, C-5), 146.8 (Val C-4′), 164.9 (Val C-7″), 165.0 (G C-7), 165.1 (G C-7), 166.6 (G C-7), 167.4, 167.9, 168.6 (2C), 169.2, 169.6 (HHDP C-7, C-7′ and Val C-7, C-7′), glucose carbons, see Table III.

Acid Hydrolysis of Nobotanins A (17) and F (18) A solution of **17** (3 mg) in 5% sulfuric acid (0.8 ml) was heated in a boiling-water bath for 5 h. After cooling, the reaction mixture was extracted with EtOAc (1 ml × 2), and the EtOAc extract was methylated with an excess of ethereal CH_2N_2. The methylated products were separated by preparative TLC (SiO_2; ligroin–$CHCl_3$–acetone, 3 : 2 : 1) to give **19a** (EI-MS *m/z* 226), **20a** (EI-MS *m/z* 450) and **22a** (EI-MS *m/z* 660). The aqueous layer was neutralized with ion exchange resin (Amberlite IRA-410), and analyzed, after trimethylsilylation, by gas liquid chromatography (GLC) (2.5% OV-1, column temperature 170 °C) to detect glucose. Nobotanin F (**18**) was similarly hydrolyzed to give the same products as described above.

Methylation of Nobotanin F (18) A mixture of **18** (40 mg), anhydrous potassium carbonate (200 mg) and dimethyl sulfate (0.3 ml) in dry acetone (13 ml) was stirred at room temperature overnight, and then refluxed for 35 h. After removal of inorganic materials by filtration, the filtrate was

purified by preparative TLC (SiO_2, ligroin–$CHCl_3$–acetone, 3 : 2 : 1) to afford **18a** (7 mg), **19a** (1 mg), **21** (1 mg), **23** (1.5 mg), and octadecamethylrugosin C (**24a**) (1 mg).

18a: ^{1}H-NMR (500 MHz, acetone-d_6) δ: 7.30, 7.34, 7.35 (each 2H, s, G), 6.60, 6.66, 6.74, 6.75, 6.77, 6.85, 7.37 (each 1H, s, HHDP and Val), 6.20 (1H, d, J=8.5 Hz, H-1), 5.30 (1H, dd, J=8.5, 9 Hz, H-2), 5.61 (1H, br t, J=9 Hz, H-3), 5.78 (1H, t, J=9 Hz, H-4), 4.17 (1H, br d, J=9 Hz, H-5), 4.83 (1H, dd, J=2, 13 Hz, H-6), 4.23 (1H, br d, J=13 Hz, H-6), 6.21 (1H, d, J=8.5 Hz, H-1′), 5.35 (1H, br t, J=9 Hz, H-2′), 5.45 (1H, t, J=9 Hz, H-3′), 5.25 (1H, br t, J=9 Hz, H-4′), 4.48 (1H, br dd, J=5.5, 9 Hz, H-5′), 5.15 (1H, dd, J=5.5, 13.5 Hz, H-6′), the other H-6′ signal is overlapped by OMe signals, 3.58, 3.59, 3.61, 3.64, 3.68, 3.70, 3.73, 3.75, 3.76, 3.77, 3.78, 3.80, 3.81, 3.90, 3.91, 4.05 (each s, 3H), 3.82, 3.83, 3.85, 3.86, 3.87 (each s, 6H), 3.85 (s, 9H) (OMe × 29).

24a: EI-MS m/z: 1356 (M^+). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 7.31 (2H, s, G), 6.50, 6.69, 6.83, 6.85, 7.25 (each 1H, s, HHDP and Val), 6.26 (1H, d, J=8.5 Hz, H-1), 5.23 (1H, dd, J=8.5, 9 Hz, H-2), 5.55 (1H, dd, J=9, 10 Hz, H-3), 5.06 (1H, t, J=10 Hz, H-4), 4.39 (1H, br dd, J=6.5, 10 Hz, H-5), 5.15 (1H, dd, J=6.5, 13.5 Hz, H-6), the other H-6 signal is overlapped by OMe signals, 3.58, 3.66, 3.70, 3.74, 3.83, 3.85, 3.86, 3.90, 4.06 (each 3H, s, OMe × 9), 3.76 (9H, s, OMe × 3), 3.80, 3.89, 3.87 (each 6H, OMe × 6).

Partial Hydrolysis of Nobotanin F (18) An aqueous solution (90 ml) of **18** (130 mg) was refluxed under a nitrogen atmosphere for 15 h, then allowed to cool. The solvent was evaporated off and the residue was subjected to column chromatography over Sephadex LH-20 with EtOH to give gallic acid (**19**) (2 mg), 2,3-(S)-hexahydroxydiphenoyl-D-glucose (**7**) (3 mg), isostrictinin (**8**) (12 mg), **25** (8 mg), **26** (4 mg) and **27** (1 mg).

25: An off-white amorphous powder, $[\alpha]_D$ +31° (c=1.0, MeOH). *Anal.* Calcd for $C_{55}H_{34}O_{34} \cdot 5H_2O$: C, 49.70: 3.34. Found: C, 49.54; H, 3.78. FAB-MS m/z: 1261 $(M+Na)^+$. UV λ_{max} (MeOH) nm (log ε): 220 (4.92), 265 (4.77), 363 (3.93). CD (MeOH) $[\theta]$ (nm): $+10.1 \times 10^4$ (235), -4.7×10^4 (262), -1.4×10^4 (310). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 6.70, 7.10 (each 2H, s, G), 6.39, 6.43 (each 1H, s, HHDP), 7.16, 7.19, 7.54 (each 1H, s, dilactonized Val), 6.18 (1H, d, J=8.5 Hz, H-1), 5.08 (1H, dd, J=8.5, 9.4 Hz, H-2), 5.41 (1H, dd, J=9.4, 9.8 Hz, H-3), 5.53 (1H, t, J=9.8 Hz, H-4), 4.27 (1H, m, H-5), 4.33 (1H, br d, J=13 Hz, H-6), 4.09 (1H, dd, J=4.4, 13 Hz, H-6).

An aqueous solution (1 ml) of **25** (1 mg) was treated with tannase at 37 °C and the reaction process was monitored by normal phase HPLC. After 2 h, **25** (t_R 3.34 min) was completely converted into **26** (t_R 2.76 min).

26: An off-white amorphous powder, $[\alpha]_D$ +35° (c=0.5, MeOH). *Anal.* Calcd for $C_{48}H_{30}O_{30} \cdot 6H_2O$: C, 48.25; H, 3.54. Found: C, 47.92; H, 3.74. UV λ_{max} (MeOH) nm (log ε): 217 (4.93), 257 (4.83), 363 (3.99). ^{1}H-NMR (400 MHz, acetone-d_6) α- and β-anomer (2:3) δ: 6.42 (2/5H, s), 6.43 (3/5H, s), 6.55 (3/5H, s), 6.56 (2/5H, s) (HHDP), 6.89 (4/5H, s), 6.90 (6/5H, s) (G), 7.13 (2/5H, s), 7.14 (3/5H, s), 7.17 (2/5H, s), 7.20 (3/5H, s), 7.53 (2/5H, s), 7.54 (3/5H, s) (dilactonized Val), 5.38 [2/5H, d, J=4.5 Hz, H-1 of α-anomer (α)], 4.97 [2/5H, dd, J=4.5, 10 Hz, H-2 (α)], 5.46 [2/5H, t, J=10 Hz, H-3 (α)], 5.39 (2/5H, t, J=10 Hz, H-4 (α)], 5.01 [3/5H, d, J=8.5 Hz, H-1 of β-anomer (β)], 4.78 [3/5H, dd, J=8.5, 10 Hz, H-2 (β)], 5.20 [3/5H, t, J=10 Hz, H-3 (β)], 5.37 [3/5H, t, J=10 Hz, H-4 (β)], 4.27 (ddd, J=2, 5, 10 Hz), 4.20 (ddd, J=4, 6, 10 Hz) [1H in total, H-5 (α and β)], 3.50 [2H, m, H-6 (α and β)].

27: A light brown amorphous powder, $[\alpha]_D$ +23° (c=0.5, MeOH). *Anal.* Calcd for $C_{41}H_{28}O_{26} \cdot 7H_2O$: C, 46.33; H, 3.98. Found: C, 46.49; H, 3.67. UV λ_{max} (MeOH) nm (log ε): 217 (4.49), 262 (4.36), 363 (3.53). ^{1}H-NMR (400 MHz, acetone-d_6) δ: 6.88, 7.11 (each 2H, s, G), 7.15, 7.24, 7.54 (each 1H, s, dilactonized Val), 5.65 (1H, d, J=8 Hz, H-1), 3.58 (1H, dd, J=8, 10 Hz, H-2), 3.68 (1H, t, J=10 Hz, H-3), 5.14 (1H, t, J=10 Hz, H-4), 4.07 (1H, dd, J=1.6, 12 Hz, H-6), 3.90 (1H, dd, J=5.5, 12 Hz, H-6), 3.84 (1H, ddd, J=1.6, 5.5, 10 Hz, H-5).

Methylation of Nobotanin A (17) A mixture of **17** (100 mg), anhydrous potassium carbonate (1 g) and dimethyl sulfate (0.8 ml) in dry acetone (10 ml) was stirred at room temperature for 24 h and then refluxed for 30 h. After removal of inorganic materials by filtration, the reaction mixture was concentrated and purified by preparative TLC (ligroin–benzene–acetone, 2 : 4 : 1; triple development) to afford the β-anomer (**1a**) (1 mg) of tridecamethylpedunculagin, octadecamethylrugosin C (**24a**) (2 mg), permethylated derivative (**5a**) (1.5 mg) of 6-O-galloyl-2,3-(S)-HHDP-glucose (**5**), and heptacosamethylnobotanin A (**17a**) (38 mg). The methylated derivatives **1a**, **24a** and **5a** were identified by direct comparison with authentic samples prepared by similar methylation.

1a: ^{1}H-NMR (400 MHz, acetone-d_6) δ: 6.60, 6.81, 6.83, 6.96 (each 1H, s, HHDP), 4.86 (1H, d, J=8 Hz, H-1), 5.30 (1H, dd, J=8, 10 Hz, H-2), 5.02 (1H, t, J=10 Hz, H-3), 4.92 (1H, t, J=10 Hz, H-4), 4.30 (1H, br dd, J=6.5, 10 Hz, H-5), 5.25 (1H, dd, J=6.5, 13 Hz, H-6), 3.99 (1H, dd, J=1.5, 13 Hz, H-6).

5a: ^{1}H-NMR (400 MHz, acetone-d_6) α- and β-anomer (*ca.* 1 : 1) δ: 7.35 (2H, s, G), 6.81, 6.85 (each s, 1H in total), 6.92, 6.93 (each s, 1H in total) (HHDP), 5.16 [1/2H, d, J=5 Hz, H-1 (α)], 5.02 (1/2H, d, J=7.5 Hz, H-1 (β)], 4.82 [1H, m, H-2 (α and β)], 5.35 [1H, t, J=9.5 Hz, H-3 (α and β)], 4.75, 4.68 [each 1/2H, dd, J=2, 12 Hz, H-6 (α and β)], 4.55, 4.52 [each 1/2H, dd, J=6.5, 12 Hz, H-6′ (α and β)], 4.08 [1H, ddd, J=2, 6.5, 9.5 Hz, H-5 (α and β)], 3.94, 3.93 [each 1/2H, t, J=9.5 Hz, H-4 (α and β)].

Heptacosamethylnobotanin A (17a) A white amorphous powder, $[\alpha]_D$ +19° (c=1.0, acetone). *Anal.* Calcd for $C_{102}H_{106}O_{48} \cdot 2H_2O$: C, 57.35; H, 5.15. Found: C, 57.26; H, 5.24. FAB-MS m/z: 2099 $(M+H)^+$. ^{1}H-NMR (500 MHz, acetone-d_6) α- and β-anomer (1 : 1) δ: 7.31, 7.28 (each s, 2H in total, G), 7.29, 7.26 (each s, 2H in total, G), 7.34, 7.25 (each s, 1H in total), 6.85, 6.84 (each s, 1H in total), 6.83, 6.82 (each s, 1H in total), 6.78 (s, 1H), 6.70, 6.69 (each s, 1H in total), 6.63, 6.60 (each s, 1H in total) (HHDP and Val), 5.08 [1/2H, d, J=3.5 Hz, H-1 of α-anomer (α)], 5.11 [1/2H, dd, J=3.5, 10 Hz, H-2 (α)], 5.57 [1/2H, t, J=10 Hz, H-3 (α)], 5.74 [1/2H, t, J=10 Hz, H-4 (α)], 4.12 [1/2H, br d, J=10 Hz, H-5 (α)], 4.78 [1/2H, d, J=8 Hz, H-1 of β-anomer (β)], 4.91 [1/2H, dd, J=8, 10 Hz, H-2 (β)], 5.30 [1/2H, t, J=10 Hz, H-3 (β)], 5.64 [1/2H, t, J=10 Hz, H-4 (β)], 3.93 [overlapped by OMe signals, H-5 (β)], 4.27, 4.33, 4.84, 4.88 [each 1/2H, br d, J=13 Hz, H-6 (α and β)], 6.23, 6.24 [each 1/2H, d, J=8.5 Hz, H-1′ (α and β)], 5.26, 5.38 [each 1/2H, dd, J=8.5, 9 Hz, H-2′ (α and β)], 5.49, 5.50 [each 1/2H, dd, J=9, 10 Hz, H-3′ (α and β)], 5.15, 5.16 [each 1/2H, t, J=10 Hz, H-4′ (α and β)], 4.48 [1H, dd, J=8, 10 Hz, H-5′ (α and β)], 5.23, 5.18 [H-6′ (α and β), overlapped by H-2′ and H-4′], 3.83 [H-6′ (α and β) overlapped by OMe signals].

Partial Hydrolysis of Nobotanin A (17) An aqueous solution (80 ml) of **17** (100 mg) was refluxed in a nitrogen atmosphere for 23 h, then allowed to cool. The solvent was evaporated off, and the residue was submitted to column chromatography over Sephadex LH-20 with EtOH to give gallic acid (**19**) (6 mg), isostrictinin (**8**) (11 mg) and **26** (5 mg).

Hydrolysis of Nobotanin F (18) with Tannase A solution (30 ml) of **18** (100 mg) was incubated with tannase (10 drops) at 37 °C overnight. The reaction mixture was concentrated, acidified with diluted HCl and then chromatographed on Sephadex LH-20 (EtOH→EtOH–MeOH) to yield **19** and a degalloylated product, which was identified as nobotanin A (**17**) by HPLC and ^{1}H-NMR spectral comparisons with an authentic sample.

Acknowledgements The authors are grateful to Prof. Y. Asakawa and Dr. Y. Shiobara, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, for measuring some of the NMR spectra, and Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University, for CD spectral measurements. Thanks are also due to Mr. S. Iwadow for measuring mass spectra.

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Triazole Antifungals. III.[1] Stereocontrolled Synthesis of an Optically Active Triazolylmethyloxirane Precursor to Antifungal Oxazolidine Derivatives

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Stereocontrolled synthesis of an optically active triazolylmethyloxirane 2, an important intermediate for the preparation of antifungal oxazolidine compounds 1, was achieved by two methods using L-lactic acid as a starting material. The key intermediate ketone 6 used in the procedures also served for the synthesis of the enantiomer of 2 and the corresponding diastereomeric epoxide.

Keywords stereocontrolled synthesis; triazole antifungal; L-lactic acid; chiral epoxide; (triazolylmethyl)oxirane; stereoselective epoxidation; (dimethylisopropoxysilyl)methylmagnesium chloride; Mitsunobu reaction

In a previous paper[1] we reported the synthesis and antifungal activities of 3-acyl-5-aryl-4-methyloxazolidines (**1**), which were designed as potential inhibitors of the fungal cytochrome P-450 14α-demethylase. These compounds exhibited remarkably potent activity against a mouse systemic *Candida albicans* infection after oral or parenteral administration. Comparison of the activities of both enantiomers of the 2,4-dichlorophenyl analog (**1**: X=2,4-Cl_2) revealed that the antifungal activity resides mainly in the (4*R*,5*R*) enantiomer. The potent activity of these triazoles was hypothesized to be a consequence of a structural similarity between (4*R*,5*R*)-**1** and lanosterol, a substrate of the cytochrome P-450 14α-demethylase. The above-described optically active 2,4-(dichlorophenyl)oxazolidines were obtained *via* optical resolution of the racemic intermediate using *d*- or *l*-10-camphorsulfonic acid as a resolving agent. In order to extend our evaluation studies on this series of compounds, we needed the corresponding (2,4-difluorophenyl)oxazolidines in optically active forms. However, several attempts to resolve the racemic 2,4-difluorophenyl analogs in a practical manner have failed.[2] In this paper we report stereoselective synthesis of an optically active triazolylmethyloxirane (2*R*,3*S*)-**2** and related stereoisomers from a common starting material. The epoxide **2** is an important intermediate to prepare the (4*R*,5*R*)-(2,4-difluorophenyl)oxazolidines (**1**: X=2,4-F_2) *via* another key intermediate, the azidoalcohol (2*R*,3*R*)-**3**.

Stereoselective synthesis of (2*R*,3*S*)-**2** and its enantiomer was performed as shown in Chart 2 starting from (*S*)-2-acetoxypropionic acid (**4**), which was readily provided by acetylation of L-lactic acid according to the literature procedure.[3] Friedel–Crafts reaction of the acyl chloride, derived from (*S*)-**4**, and 1,3-difluorobenzene afforded a 1 : 1 mixture of α-acetoxy- and α-hydroxy-propiophenone, (*S*)-**5** and (*S*)-**6**, which, on acid treatment in methanol (MeOH), gave the pure alcohol (*S*)-**6**, $[\alpha]_D^{25}$ −67.1° (*c*=1.17, $CHCl_3$), in 67% yield from (*S*)-**4**. The optical purity of (*S*)-**6** was determined to be >99.5% ee by high-performance liquid chromatographic (HPLC) analysis using a chiral stationary phase column. For the synthesis of (2*R*,3*S*)-**2**, inversion at the asymmetric center in (*S*)-**6** was required. Tosylation of (*S*)-**6** with *p*-toluenesulfonyl chloride in pyridine at −10 °C afforded the crude solid, which was purified by recrystallization to give the optically pure tosylate (*S*)-**7** in 60% yield. In this reaction, a racemic α-chloropropiophenone derivative was obtained as a by-product. To avoid the formation of this by-product, *p*-toluenesulfonic anhydride was used instead of *p*-toluenesulfonyl chloride, and the yield of (*S*)-**7** was improved to 76%. On the other hand, mesylation of (*S*)-**6** with methanesulfonyl chloride and triethylamine in CH_2Cl_2 resulted in the competing formation of the sultone[4] **10** in 25% yield along with a 63% yield of the mesylate (*S*)-**8**. The S_N2 displacement reaction of the tosylate (*S*)-**7** to (*R*)-**6** was conducted by slow addition (over a period of 2 h) of an aqueous solution of 1 eq of lithium hydroxide to (*S*)-**7** in *N*,*N*-dimethylformamide (DMF) at −15°C. The enantiomeric hydroxyketone (*R*)-**6**, $[\alpha]_D^{25}$ +64.2° (*c*=0.74, $CHCl_3$), was obtained in 73% yield. The product was partially racemized (determined by HPLC to be 94.5% ee). Faster addition of lithium hydroxide or a higher reaction temperature brought about appreciable racemization and lowered the optical purity of the product.

Protection of the hydroxy group of (*R*)-**6** with the tetrahydropyranyl group yielded (*R*)-**9**, which was stereoselectively transformed into the chiral epoxide (2*R*,3*S*)-**2** according to the procedure described in the previous paper for the preparation of (±)-**2** from (±)-**9**.[1] The ketone (*R*)-**9** was treated with the Grignard reagent [(dimethylisopropoxysilyl)methylmagnesium chloride] developed by Tamao and Ishida,[5] and the resulting silylalcohol **11** was oxidatively desilylated[5] and then hydrolyzed to the optically pure triol

1 : X=halogen (s) 2 3 → 1 (X=2, 4-F_2)

Chart 1

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(S)-4 → (S)-5 : R=Ac, (S)-6 : R=H → (S)-7 : R=p-tolyl (–C₆H₄–Me), (S)-8 : R=Me → (R)-6 : R=H, (R)-9 : R=THP

10

(R)-9 → 11 → (2R, 3R)-12 → 13 → [14] → (2R, 3S)-2 → (2R, 3R)-3

(S)-6 → (S)-9 → (2S, 3S)-12 → (2S,3R)-2 → (2S, 3S)-3

Ar : 2, 4-difluorophenyl

Chart 2

(S)-9 → 15 → (S)-16 → (2S, 3R) 17 → (2R, 3R) 18 : R=COPh, 19 : R=H → 14 → (2R, 3S)-2

17 → 20 → (2R, 3R)-21 → (2R, 3S)-22

17 → 23 → 24 : n=1, 25 : n=2 → (2R, 2R)-3

Ar : 2, 4-difluorophenyl

Chart 3

(2*R*,3*R*)-**12**, after recrystallization, in 77% overall yield from (*R*)-**9**. Mesylation of (2*R*,3*R*)-**12** gave the dimesylate **13**, which was then treated with excess sodium triazolide to afford the epoxide (2*R*,3S)-**2**, mp 88—89.5 °C, $[\alpha]_D^{25}$ −7.55° (c=1.06, $CHCl_3$), in 76% yield from (2*R*,3*R*)-**12**. This reaction is known to proceed *via* an intermediate epoxymesylate **14**, which affords the epoxide **2** through ring-opening and recyclization by an attack of the triazolide ion.[1] The epoxide (2*R*,3*S*)-**2** gave the azidoalcohol (2*R*,3*R*)-**3** by reaction with sodium azide in a usual manner.

The enantiomeric epoxide (2*S*,3*R*)-**2** was also prepared starting from (*S*)-**6**, *via* (*S*)-**9** and (2*S*,3*S*)-**12**, following the

same reaction sequence as described for the (2*R*,3*S*) epoxide **2**. This epoxide was transformed into the azidoalcohol enantiomer (2*S*,3*S*)-**3**.

Alternative synthesis of the epoxide (2*R*,3*S*)-**2** from the ketone (*S*)-**6** was achieved as shown in Chart 3. As can be seen from the above results, the epoxymesylate **14**, a reaction intermediate in the transformation of **13** to (2*R*,3*S*)-**2**, should be a useful synthetic intermediate. Stereoselective synthesis of the corresponding epoxyalcohol (2*R*,3*R*)-**19** from (*S*)-**9** was carried out by means of a reaction sequence including the silyl-Wittig olefination, stereoselective epoxidation and Mitsunobu reaction. The Grignard reaction of (*S*)-**9** with (trimethylsilyl)methylmagnesium chloride[6] afforded the silylalcohol **15**, which, on treatment with a catalytic amount of *p*-toluenesulfonic acid in MeOH, underwent β-elimination and deprotection to afford the allylic alcohol (*S*)-**16** in 92% yield from (*S*)-**9**. Epoxidation of (*S*)-**16** with *m*-chloroperbenzoic acid gave an inseparable 4:1 mixture of the epoxyalcohols (2*S*,3*R*)-**17** and (2*S*,3*S*)-**19**, whereas the reaction of (*S*)-**16** with *tert*-butyl hydroperoxide and a catalytic amount of oxyvanadium acetylacetonate[7] proceeded with higher stereoselectivity to provide a *ca.* 20:1 mixture of (2*S*,3*R*)-**17** and (2*S*,3*S*)-**19** in 87% yield. The latter mixture was subjected to Mitsunobu reaction,[8] and the resulting benzoate **18** (96% yield) was solvolyzed in MeOH in the presence of a catalytic amount of MeONa to give (2*R*,3*R*)-**19** (85% yield). This product was contaminated with a small amount (<5%) of the diastereoisomer (2*R*,3*S*)-**17**. Mesylation of this epoxyalcohol followed by treatment of the resulting mesylate **14** with sodium triazolide afforded the epoxide (2*R*,3*S*)-**2**, in 77% yield (after recrystallization) from (2*R*,3*R*)-**19**; this product was identical with the aforementioned optically pure (2*R*,3*S*) epoxide.

The diastereomeric epoxide (2*R*,3*R*)-**21** was also obtained as an oil in a similar manner from the epoxymesylate **20**, which was prepared by mesylation of the epoxyalcohol (2*S*,3*R*)-**17**. This oily epoxide was transformed into the crystalline azidoalcohol (2*R*,3*S*)-**22**.

A different approach to the azidoalcohol (2*R*,3*R*)-**3** from the above-described epoxyalcohol **17** was also examined. Recently, Sharpless and coworkers reported the usefulness of vicinal diol cyclic sulfates,[9] which act as reactive epoxide equivalents. Thus, the (2*S*,3*R*) epoxyalcohol **17** was treated with sodium triazolide to afford the diol **23**, which was converted to **24**, then to the cyclic sulfate **25** according to the Sharpless procedure. Treatment of **25** with sodium azide in DMF, followed by hydrolysis of the resulting sulfate ester, gave the desired azidoalcohol (2*R*,3*R*)-**3**. The yield, however, was unsatisfactory (<30%).

Conversion of the above-mentioned optically active triazolylmethyloxiranes and azidoalcohols to new aminoalcohol derivatives with antifungal activity will be reported in a forthcoming paper.

Experimental

Melting points are uncorrected. Infrared spectra (IR) were recorded on a JASCO A-2 spectrometer and proton magnetic resonance spectra (^{1}H-NMR) on a Varian EM-360L spectrometer in $CDCl_3$ using Me_4Si as an internal standard. Mass spectra (MS) were obtained on a JEOL JMS D300 spectrometer. Optical rotations were determined on a Perkin-Elmer 141 spectrometer. Thin-layer chromatography (TLC) was performed on TLC plates, Silica gel 60 F_{254} precoated, layer thickness 0.25 mm (E. Merck), and spots were made visible by ultraviolet (UV) irradiation, by spraying with vanadic acid–sulfuric acid followed by heating, or by iodine treatment. Chromatography columns were prepared with silica gel (60—110 mesh, Kanto Chemical Co., Inc.) and preparative TLC was carried out on TLC plates, Silica gel 60 F_{254} precoated, layer thickness 2 mm (E. Merck). The amount of silica gel and the developing solvents are shown in parentheses. The abbreviations used are as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; td, triplet of doublets; q, quartet; qd, quartet of doublets; m, multiplet; br, broad.

(*S*)-2-Hydroxy-2′,4′-difluoropropiophenone [(*S*)-6] Oxalyl chloride (16.9 ml, 0.197 mol) and then DMF (1.0 ml) was added to a solution of (*S*)-2-acetoxypropionic acid (**4**) [$[\alpha]_D^{25}$ −49.0° (c=1.35, $CHCl_3$), lit.,[3] $[\alpha]_D^{22}$ −49.3° (c=7.3, $CHCl_3$); 20.0 g, 0.15 mol] in CH_2Cl_2 (100 ml). The mixture was stirred at room temperature for 1.5 h. At the end of this time, the solvent was distilled off under reduced pressure. To the oily residue, 1,3-difluorobenzene (25.9 g, 0.227 mol) and aluminum chloride (50.5 g, 0.379 mol) were added, with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature and stirring was continued overnight. The mixture was diluted with CH_2Cl_2 (60 ml), and poured into ice-water with stirring, then the separated oil was extracted with AcOEt (400 ml). The extract was washed, in turn, with aqueous $NaHCO_3$ and with brine. Evaporation of the solvent *in vacuo* afforded a *ca.* 1:1 mixture (36 g) of (*S*)-**5** [^{1}H-NMR ($CDCl_3$) δ: 1.48 (3H, dd, J=7, 1.5 Hz), 2.10 (3H, s), 5.76 (1H, qd, J=7, 1.5 Hz), 6.7—7.2 (2H, m), 7.7—8.2 (1H, m)] and (*S*)-**6**. The mixture was dissolved in cold MeOH (180 ml), 45% (v/v) H_2SO_4 (30 ml) was added, and the whole was allowed to stand at room temperature overnight. The mixture was concentrated under reduced pressure to 1/3 the initial volume, and partitioned between AcOEt and water. The organic layer was washed successively with aqueous $NaHCO_3$ and brine. Evaporation of the solvent and distillation of the product gave (*S*)-**6** (18.6 g, 67%), bp 65—67 °C (3 mmHg), $[\alpha]_D^{25}$ −67.1° (c=1.17, $CHCl_3$). The optical purity was >99.5% ee, as determined by HPLC using a chiral stationary phase column, Chiralcel OJ® (4.6 mm i.d. × 250 mm, Daicel Chemical Industries, Tokyo, Japan) (column temperature, 23 °C; mobile phase, hexane–isopropanol, 99:1, v/v; flow rate, 1.0 ml/min; detection, UV at 254 nm). Retention times of the enantiomers are as follows: (*S*)-**6**, 13.2 min; (*R*)-**6**, 14.4 min. *Anal.* Calcd for $C_9H_8F_2O_2$: C, 58.07; H, 4.33; F, 20.41. Found: C, 58.18; H, 4.35; F, 20.32. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3510, 1678, 1615. ^{1}H-NMR ($CDCl_3$+D_2O) δ: 1.42 (3H, dd, J=6.5, 2 Hz), 5.00 (1H, qd, J=6.5, 2 Hz), 6.7—7.3 (2H, m), 7.8—8.2 (1H, m).

(*S*)-2-(*p*-Toluenesulfonyloxy)-2′,4′-difluoropropiophenone [(*S*)-7] i) *p*-Toluenesulfonyl chloride (10.24 g, 53.7 mmol) was added to a solution of (*S*)-**6** (5.00 g, 26.9 mmol) in pyridine (25 ml) at −10 °C and the whole was stirred at the same temperature for 6 h. The mixture was partitioned between AcOEt and aqueous $NaHCO_3$. The organic layer was washed with brine, dried and concentrated *in vacuo* to give a crystalline residue, which was recrystallized from acetone–cyclohexane to give (*S*)-**7** (5.49 g, 60%), mp 88—90 °C, $[\alpha]_D^{25}$ −19.3° (c=0.92, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{14}F_2O_4S$: C, 56.47; H, 4.15; S, 9.42. Found: C, 56.74; H, 4.06; S, 9.63. IR $\nu_{max}^{CHCl_3}$: 1695 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53 (3H, dd, J=7, 2 Hz), 2.42 (3H, s), 5.67 (1H, q, J=7 Hz), 6.7—7.1 (2H, m), 7.27 (2H, d, J=9 Hz), 7.78 (2H, d, J=9 Hz), 7.6—8.1 (1H, m).

The mother liquor was concentrated and chromatographed. Elution with benzene afforded 2-chloro-2′,4′-difluoropropiophenone (380 mg, 7%) as an oil, whose optical rotation was 0°. MS m/z: 206, 204 (M^+). IR $\nu_{max}^{CHCl_3}$: 1690, 1610. ^{1}H-NMR ($CDCl_3$) δ: 1.70 (3H, d, J=6.5 Hz), 5.15 (1H, q, J=6.5 Hz), 6.7—7.2 (2H, m), 7.75—8.25 (1H, m).

ii) A solution of (*S*)-**6** (340 mg, 1.83 mmol) and *p*-toluenesulfonic anhydride (895 mg, 2.74 mmol) in pyridine (1.4 ml) was allowed to stand at 0°C for 30 min. The mixture was diluted with AcOEt and washed, in turn, with aqueous $NaHCO_3$ and with brine. Evaporation of the solvent under reduced pressure and purification of the product by column chromatography (10 g, benzene) afforded a crystalline mass, which was recrystallized from acetone–hexane to furnish (*S*)-**7** (472 mg, 76%), mp 88—90 °C.

Methanesulfonylation of (*S*)-6 Methanesulfonyl chloride (95 mg, 0.83 mmol) was added, with stirring at 0 °C, to a solution of (*S*)-**6** (118 mg, 0.63 mmol) and triethylamine (84 mg, 0.83 mmol) in CH_2Cl_2 (3.5 ml). After 10 min, the mixture was diluted with AcOEt, washed with brine and dried. Evaporation of the solvent under reduced pressure followed by column chromatography (AcOEt–benzene, 1:15, v/v) of the product gave the mesylate (*S*)-**8** (105 mg, 63%) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 1.61 (3H, dd, J=7, 1.5 Hz), 3.15 (3H, s), 5.87 (1H, qd, J=7, 1 Hz), 6.7—7.2 (2H, m), 7.8—8.2 (1H, m). Further elution afforded a more polar product **10** (38 mg, 25%), mp 119—121 °C (colorless prisms from AcOEt–hexane). IR

ν_{max}^{KBr}: 3490 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (3H, d, J=6.5 Hz), 3.61 (1H, dd, J=14, 1.5 Hz), 3.98 (1H, s), 4.15 (1H, d, J=14 Hz), 5.16 (1H, q, J=6.5 Hz), 6.7—7.2 (2H, m), 7.79 (1H, td, J=9, 6 Hz).

(*R*)-2-Hydroxy-2′,4′-difluoropropiophenone [(*R*)-6] A solution of lithium hydroxide (352 mg, 14.7 mmol) in H_2O (20 ml) was added, with stirring at −15°C, to a solution of (*S*)-**7** (5.00 g, 14.7 mmol) in DMF (50 ml) over a period of 2 h. The mixture was stirred at the same temperature for 1 h, then a 5% aqueous solution of NH_4Cl (20 ml) was added. The whole was extracted with AcOEt, washed with brine and concentrated *in vacuo* to leave an oil, which was purified by column chromatography on silica gel (50 g, benzene–AcOEt, 20:1, v/v) to give (*R*)-**6** (2.00 g, 73%) as an oil, $[\alpha]_D^{25}$ +64.2° (c=0.74, $CHCl_3$), whose ^{1}H-NMR and TLC behavior were identical with those of (*S*)-**6** described above. The optical purity was determined as 94.5% ee by HPLC in the same manner as described for (*S*)-**6**.

(*R*)-2-(2-Tetrahydropyranyloxy)-2′,4′-difluoropropiophenone [(*R*)-9] A solution of (*R*)-**6** (2.00 g, 10.7 mmol) obtained above, 2,3-dihydropyran (1.17 g, 13.9 mmol) and pyridinium *p*-toluenesulfonate (135 mg, 0.54 mmol) in CH_2Cl_2 (20 ml) was stirred overnight at room temperature. The mixture was washed successively with aqueous $NaHCO_3$ and brine, dried, and concentrated under reduced pressure to give an oily residue, which was chromatographed (40 g, benzene–hexane, 5:1, v/v) to provide (*R*)-**9** (2.54 g, 87%) as an oil, $[\alpha]_D^{25}$ +53.4° (c=1.24, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$: 1695 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (1.5H, dd, J=6.5, 3 Hz), 1.45 (1.5H, dd, J=6.5, 1.5 Hz), 1.3—2.0 (6H, m), 3.1—4.1 (2H, m), 4.6—4.8 (1H, m), 4.86 (0.5H, qd, J=6.5, 2 Hz), 5.10 (0.5H, dq, J=6.5, 2 Hz), 6.7—7.2 (2H, m), 7.7—8.2 (1H, m).

(2*S*,3*R*)-2-(2,4-Difluorophenyl)-1-(dimethylisopropoxysilyl)-3-(2-tetrahydropyranyloxy)-2-butanol (11) A suspension of a small amount of magnesium activated with methyl iodide in ether was added, under an N_2 atmosphere, to a refluxing mixture of magnesium (0.50 g, 20.6 mmol), chloromethyldimethylisopropoxysilane[5] (3.03 g, 18.2 mmol) and ether (60 ml) to initiate the reaction. The mixture was heated under reflux for 3 h and then cooled to 0°C. A solution of (*R*)-**9** (2.62 g, 9.70 mmol) in tetrahydrofuran (THF) (15 ml) was added dropwise, with stirring at 0°C, to the above mixture over a period of 10 min. The whole was warmed to room temperature, stirred for 15 min and again cooled to 0°C. A saturated aqueous solution of NH_4Cl was added to the mixture, which was extracted with AcOEt. The extract was washed with brine and concentrated *in vacuo* to give **11** (4.02 g) as an oil, which was used without further purification for the next reaction. IR $\nu_{max}^{CHCl_3}$: 3425 cm^{-1}.

(2*R*,3*R*)-2-(2,4-Difluorophenyl)-1,2,3-butanetriol [(2*R*,3*R*)-12] Sodium hydrogen carbonate (0.70 g, 8.33 mmol) and 35% aqueous H_2O_2 (7.0 ml) were added to a solution of **11** (4.54 g, 11.28 mmol) in THF–MeOH (1:1, v/v, 50 ml), and the whole was heated at 70°C for 1.5 h. The mixture was cooled, diluted with AcOEt (100 ml) and washed with brine. Evaporation of the solvent under reduced pressure gave an oily product (4.50 g), whose IR and ^{1}H-NMR spectra were identical with those of the corresponding racemate.[1] The crude product (2.85 g) was treated with *p*-toluenesulfonic acid (120 mg, 0.63 mmol) in MeOH (40 ml) at room temperature for 30 min. After the addition of triethylamine (0.071 g, 0.70 mmol), the mixture was concentrated under reduced pressure to give a crystalline mass, which was recrystallized from acetone–cyclohexane to yield (2*R*,3*R*)-**12** [1,58 g, 77% yield from (*R*)-**9**], mp 81—82°C, $[\alpha]_D^{25}$ +11.4° (c=1.11, $CHCl_3$). *Anal.* Calcd for $C_{10}H_{12}F_2O_3$: C, 55.04; H, 5.55; F, 17.42. Found: C, 55.08; H, 5.66; F, 17.21. IR ν_{max}^{KBr} cm^{-1}: 3430 (br), 3370 (br). The ^{1}H-NMR spectrum of (2*R*,3*R*)-**12** was identical with that of the corresponding racemate.[1]

(2*R*,3*R*)-2-(2,4-Difluorophenyl)-1,3-bis(methanesulfonyloxy)-2-butanol (13) Methanesulfonyl chloride (1.51 ml, 19.5 mmol) was added, with stirring at 0°C, to a solution of (2*R*,3*R*)-**12** (1.54 g, 7.06 mmol) in pyridine (6 ml). Stirring was continued at the same temperature for 30 min, then the solvent was distilled off under reduced pressure to leave an oil, which was dissolved in AcOEt. This solution was washed, in turn, with aqueous $NaHCO_3$ and with brine. Evaporation of the solvent *in vacuo* gave **13** (2.69 g, 96%) as an oil, which was used without further purification for the next reaction. The ^{1}H-NMR spectrum of **13** was identical with that of the corresponding racemate.[1]

(2*R*,3*S*)-2-(2,4-Difluorophenyl)-3-methyl-2-[(1*H*-1,2,4-triazol-1-yl)-methyl]oxirane [(2*R*,3*S*)-2] 1*H*-1,2,4-Triazole (1.95 g, 28.2 mmol) was slowly added to a suspension of sodium hydride (55% mineral oil dispersion, 1.08 g, 24.7 mmol, washed with hexane) in DMF (30 ml), with stirring at 0°C. When hydrogen gas ceased to evolve, a solution of **13** (2.62 g, 7.05 mmol) in DMF (5 ml) was added. After being stirred at 65—70°C for 2 h, the mixture was concentrated under reduced pressure and the residue was partitioned between benzene and water. The organic layer was washed with brine and dried. Evaporation of the solvent under reduced pressure gave a crude product, which was purified by column chromatography (30 g, benzene–AcOEt, 2:1, v/v) to give a crystalline mass. Recrystallization of this from benzene–hexane gave (2*R*,3*S*)-**2** (1.40 g, 76% yield from (2*R*,3*R*)-**12**), mp 88—89.5°C, $[\alpha]_D^{25}$ −7.55° (c=1.06, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{11}F_2N_3O$: C, 57.37; H, 4.11; N, 16.73. Found: C, 57.43; H, 4.37; N, 16.86. The IR and ^{1}H-NMR spectra were identical with those of the corresponding racemate.[1]

(2*R*,3*R*)-3-Azido-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol [(2*R*,3*R*)-3] A mixture of (2*R*,3*S*)-**2** (30.0 g, 119 mmol), sodium azide (23.5 g, 360 mmol), ammonium chloride (10.6 g, 195 mmol) and DMF (400 ml) was stirred at 105°C for 4 h. The mixture was concentrated under reduced pressure and the residue was partitioned between benzene and water. The organic layer was washed with brine, dried, and evaporated *in vacuo*. The crystalline residue was recrystallized from benzene–hexane to give (2*R*,3*R*)-**3** (25.8 g, 73%), mp 139—140°C, $[\alpha]_D^{25}$ −88° (c=1.12, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{12}F_2N_6O$: C, 48.98; H, 4.11; F, 12.91; N, 28.56. Found: C, 48.92; H, 4.08; F, 13.02; N, 28.47. The IR and ^{1}H-NMR spectra were identical with those of the corresponding racemate.[1]

(*S*)-2-(2-Tetrahydropyranyloxy)-2′,4′-difluoropropiophenone [(*S*)-9] According to a procedure similar to that described for (*R*)-**9**, (*S*)-**9** (13.0 g, 90%) was prepared as an oil from (*S*)-**6** (10.0 g). $[\alpha]_D^{25}$ −56.3° (c=1.11, $CHCl_3$). The IR and ^{1}H-NMR spectra were identical with those of (*R*)-**9**.

(2*S*,3*S*)-2-(2,4-Difluorophenyl)-1,2,3-butanetriol [(2*S*,3*S*)-12] According to a procedure similar to that described for (2*R*,3*R*)-**12**, (2*S*,3*S*)-**12** was prepared in 79% yield from (*S*)-**9** as crystals, mp 81—82°C, $[\alpha]_D^{25}$ −11.2° (c=0.97, $CHCl_3$).

(2*S*,3*R*)-2-(2,4-Difluorophenyl)-3-methyl-2-[(1*H*-1,2,4-triazol-1-yl)-methyl]oxirane [(2*S*,3*R*)-2] According to a procedure similar to that described for (2*R*,3*S*)-**2**, (2*S*,3*R*)-**2** was prepared in 80% yield from (2*S*,3*S*)-**12** as crystals, mp 88—88.5°C, $[\alpha]_D^{25}$ +7.53° (c=1.33, $CHCl_3$).

(2*S*,3*S*)-3-Azido-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol [(2*S*,3*S*)-3] According to a procedure similar to that described for (2*R*,3*R*)-**3**, (2*S*,3*S*)-**3** was prepared in 75% yield from (2*S*,3*R*)-**2** as crystals, mp 139—140°C, $[\alpha]_D^{25}$ +88° (c=0.95, $CHCl_3$).

(*S*)-3-(2,4-Difluorophenyl)-3-buten-2-ol [(*S*)-16] A solution of (*S*)-**9** (11.2 g, 41.4 mmol) in ether (50 ml) was added dropwise to a stirred solution of (trimethylsilyl)methylmagnesium chloride[6] [prepared from chloromethyltrimethylsilane (10.0 g, 82.9 mmol) and magnesium (2.01 g, 82.6 mmol) in ether (150 ml)] over a period of 10 min at 0°C. The mixture was stirred at the same temperature for 1 h, then quenched with a saturated aqueous solution of NH_4Cl, and extracted with benzene. The extract was washed with brine and evaporated *in vacuo* to give an oily product **15** (15.0 g), which was treated with *p*-toluenesulfonic acid (0.50 g) in MeOH (100 ml) at room temperature for 12 h. The mixture was concentrated under reduced pressure to *ca.* 1/4 the initial volume, and partitioned between benzene and water. The organic layer was washed with brine and dried. Evaporation of the solvent and distillation of the product under reduced pressure gave (*S*)-**16** (7.00 g, 92%), bp 49—51°C (1.5 mmHg), $[\alpha]_D^{25}$ +49° (c=1.22, $CHCl_3$). *Anal.* Calcd for $C_{10}H_{10}F_2O$: C, 65.21; H, 5.47; F, 20.63. Found: C, 65.44; H, 5.28; F, 20.51. MS m/z: 184 (M^+), 169, 141 (100%), 140, 139, 127, 119, 114, 101, 63, 45, 43. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3430 (br). ^{1}H-NMR ($CDCl_3$) δ: 1.28 (3H, d, J=6.5 Hz), 2.1 (1H, br), 4.69 (1H, br q, J=6.5 Hz), 5.17 (1H, br s), 5.54 (1H, t, J=1.5 Hz), 6.6—7.6 (3H, m).

(2*S*,3*R*)-3-(2,4-Difluorophenyl)-3,4-epoxy-2-butanol [(2*S*,3*R*)-17] i) A solution of (*S*)-**16** (7.00 g, 38.0 mmol), *tert*-butyl hydroperoxide (70% purity, 9.80 g, 76.0 mmol) and oxyvanadium acetylacetonate (140 mg) in benzene (35 ml) was stirred at room temperature overnight. The mixture was washed with brine, dried and concentrated *in vacuo* to give an oily residue, which was chromatographed on silica gel (100 g, AcOEt–hexane, 1:3, v/v) to afford (2*S*,3*R*)-**17** (6.58 g, 87%) as an oil, $[\alpha]_D^{25}$ −26.4° (c=1.25, $CHCl_3$). This product contained a small amount of the inseparable diastereoisomer (2*S*,3*S*)-**19**; the diastereomeric ratio determined from the ^{1}H-NMR spectrum was *ca.* 20:1. MS m/z: 200 (M^+), 183, 170, 156, 155, 141, 127 (100%), 114, 101, 63, 45, 43. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560, 3500 (br). ^{1}H-NMR ($CDCl_3$) δ: 1.19 (3H, dd, J=6.5, 1.5 Hz), 2.4 (1H, br), 2.89 (1H, d, J=5 Hz), 3.26 (1H, d, J=5 Hz), 4.10 (1H, q, J=6.5 Hz), 6.6—7.1 (2H, m), 7.2—7.6 (1H, m).

ii) A solution of (*S*)-**16** (26 mg, 0.14 mmol) and *m*-chloroperbenzoic acid (85% purity, 40 mg, 0.20 mmol) in CH_2Cl_2 (1 ml) was stirred at room temperature overnight. The mixture was treated with aqueous Na_2SO_3 and extracted with AcOEt. The organic layer was washed successively with aqueous $NaHCO_3$ and brine. Evaporation of the solvent followed by purification of the crude product by preparative TLC (AcOEt–hexane, 1:3, v/v) afforded a mixture of diastereoisomers (2*S*,3*R*)-**17** and (2*S*,3*S*)-**19**

(13 mg, 46%), whose ratio was determined as 4 : 1 on the basis of the ^{1}H-NMR spectrum.

(2*R*,3*R*)-3-(2,4-Difluorophenyl)-3,4-epoxy-2-butyl Benzoate [(2*R*,3*R*)-18] Diethyl azodicarboxylate (8.48 g, 49.0 mmol) was added, with stirring at 0 °C, to a solution of triphenylphosphine (12.78 g, 48.8 mmol), benzoic acid (7.94 g, 65.0 mmol) and (2*S*,3*R*)-**17** (6.50 g, 32.5 mmol), prepared as described above by procedure i), in THF (90 ml) over a period of 5 min. The mixture was then warmed to room temperature, stirred for 1 h, and partitioned between benzene and water. The organic layer was washed with brine and dried. Evaporation of the solvent under reduced pressure gave an oily residue, which was chromatographed on silica gel (200 g, AcOEt–hexane, 1 : 10, v/v) to afford (2*R*,3*R*)-**18** (10.0 g, 96%) as an oil, $[\alpha]_D^{25}$ −52.6° (c=1.33, $CHCl_3$). This product contained a small amount (<5% by ^{1}H-NMR) of the (2*R*,3*S*) diastereoisomer, possibly derived from (2*S*,3*S*)-**19**. IR $\nu_{max}^{CHCl_3}$ 1716 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.35 (3H, dd, J=6.5, 1.5 Hz), 2.88 (1H, d, J=4.5 Hz), 3.22 (1H, d, J=4.5 Hz), 5.44 (1H, q, J=6.5 Hz), 6.6—7.1 (2H, m), 7.2—7.8 (4H, m), 7.9—8.2 (2H, m).

(2*R*,3*R*)-3-(2,4-Difluorophenyl)-3,4-epoxy-2-butanol [(2*R*,3*R*)-19] The benzoate (2*R*,3*R*)-**18** (8.40 g, 26.2 mmol) obtained above was added to a 0.5% solution of MeONa in MeOH (85 ml). The mixture was allowed to stand at room temperature for 6 h, then diluted with AcOEt, washed with brine and dried. The solvent was evaporated off *in vacuo* to give an oil, which was purified by column chromatography (100 g, AcOEt–hexane, 1 : 4, v/v) to yield (2*R*,3*R*)-**19** (4.47 g, 85%) as an oil. $[\alpha]_D^{25}$ −54.9° (c=1.45, $CHCl_3$). This product was contaminated with a small amount (<5% by ^{1}H-NMR) of the diastereoisomer (2*R*,3*S*)-**17**. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3590, 3470 (br). ^{1}H-NMR ($CDCl_3$) δ: 1.15 (3H, dd, J=6.5, 1.5 Hz), 2.2 (1H, br), 2.79 (1H, d, J=5 Hz), 3.28 (1H, d, J=5 Hz), 4.15 (1H, br), 6.6—7.1 (2H, m), 7.2—7.7 (1H, m).

(2*R*,3*R*)-3-(2,4-Difluorophenyl)-3,4-epoxy-2-butyl Methanesulfonate (14) Triethylamine (3.40 g, 33.6 mmol) and then methanesulfonyl chloride (3.35 g, 29.2 mmol) were added to a stirred solution of (2*R*,3*R*)-**19** (4.50 g, 22.5 mmol) in CH_2Cl_2 (45 ml) at −15 °C. Stirring was continued for 10 min at the same temperature, then the mixture was partitioned between AcOEt and water. The organic layer was dried and concentrated *in vacuo* to give **14** (6.33 g, 100%) as an oil, which was used without further purification for the next reaction. The ^{1}H-NMR data were identical with that of the racemic compound described previously.[1]

Transformation of the Epoxymesylate 14 into the Triazolylmethyloxirane (2*R*,3*S*)-2 The mesylate **14** (5.80 g, 20.8 mmol) in DMF (25 ml) was added to a solution of sodium triazolide, prepared from triazole (5.05 g, 73.1 mmol) and sodium hydride (55% mineral oil dispersion, 2.74 g, 62.8 mmol, washed with hexane) in DMF (50 ml), and the mixture was stirred at 60 °C for 3 h. After cooling, the mixture was diluted with benzene and washed with brine. The solvent was then distilled off under reduced pressure to give an oily residue, which was purified by column chromatography (80 g, AcOEt–hexane, 2 : 1, v/v) to afford a crystalline product (4.22 g), $[\alpha]_D^{25}$ −6.95° (c=1.41, $CHCl_3$). Recrystallization from benzene–hexane provided the pure triazolylmethyloxirane (2*R*,3*S*)-**2** [4.01 g, 77% yield from (2*R*,3*R*)-**19**], mp 88—89.5 °C, $[\alpha]_D^{25}$ −7.52° (c=1.21, $CHCl_3$).

(2*S*,3*R*)-3-(2,4-Difluorophenyl)-3,4-epoxy-2-butyl Methanesulfonate (20) Methanesulfonyl chloride (90 mg, 0.79 mmol) was added to a stirred solution of (2*S*,3*R*)-**17** (110 mg, 0.55 mmol), obtained by procedure i) above, and triethylamine (85 mg, 0.85 mmol) in CH_2Cl_2 (2 ml) at −10 °C. After 10 min, the mixture was treated with aqueous $NaHCO_3$ and extracted with AcOEt. The extract was washed with brine, dried and concentrated *in vacuo* to dryness to give the crude mesylate **20** (155 mg, *ca.* 100%) as a solid, which was used without further purification for the next reaction. ^{1}H-NMR ($CDCl_3$) δ: 1.40 (3H, dd, J=6.5, 1 Hz), 2.90 (1H, d, J=5 Hz), 2.93 (3H, s), 3.21 (1H, d, J=5 Hz), 4.99 (1H, q, J=6.5 Hz), 6.6—7.7 (3H, m).

(2*R*,3*R*)-2-(2,4-Difluorophenyl)-3-methyl-2-[(1*H*-1,2,4-triazol-1-yl)-methyl]oxirane [(2*R*,3*R*)-21] The mesylate **20** (155 mg, 0.55 mmol) in DMF (1 ml) was added to a solution of sodium triazolide, prepared from sodium hydride (55% mineral oil dispersion, 50 mg, 1.15 mmol) and triazole (90 mg, 1.30 mmol) in DMF (2 ml) as described for (2*R*,3*S*)-**2**, and the mixture was stirred at 60 °C for 3 h under an N_2 atmosphere. The cooled mixture was partitioned between benzene and brine. The organic layer was dried, and concentrated *in vacuo*, then the residue was purified by preparative TLC (AcOEt–hexane, 3 : 1, v/v) to afford (2*R*,3*R*)-**21** (86 mg, 62% yield from **17**) as an oil, $[\alpha]_D^{25}$ −5.0° (c=0.96, $CHCl_3$), which was contaminated with a small amount (*ca.* 5% content determined by ^{1}H-NMR) of the diastereomeric epoxide (2*S*,3*R*)-**2**, which could have originated from the minor epoxyalcohol (2*S*,3*S*)-**19** in (2*S*,3*R*)-**17**. MS *m/z*: 252, 251 (M^+), 236, 188, 169, 153, 141, 119, 110, 96 (100%), 69. The ^{1}H-NMR spectrum of (2*R*,3*R*)-**21** was identical with that of the racemic material.[1]

(2*R*,3*S*)-3-Azido-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol [(2*R*,3*S*)-22] A mixture of (2*R*,3*R*)-**21** (74 mg, 0.29 mmol) obtained above, sodium azide (90 mg, 1.38 mmol), ammonium chloride (20 mg, 0.37 mmol) and DMF (1.5 ml) was stirred at 110 °C for 2.5 h. After cooling, the mixture was partitioned between AcOEt and brine. The organic layer was washed with brine, dried and concentrated *in vacuo*. Preparative TLC (AcOEt–hexane, 7 : 1, v/v) of the residue gave a crystalline mass (62 mg), mp 115—120 °C, $[\alpha]_D^{25}$ +31.3° (c=0.72, $CHCl_3$), which was recrystallized from benzene–hexane to yield (2*R*,3*S*)-**22** (55 mg, 63%) as prisms, mp 126—127.5 °C, $[\alpha]_D^{25}$ +33.5° (c=0.93, $CHCl_3$). The IR and ^{1}H-NMR spectra were identical with those of the racemic material.[1]

(2*R*,3*S*)-2-(2,4-Difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-2,3-butanediol (23) The epoxyalcohol **17** (125 mg, 0.63 mmol), prepared as described above by procedure i), was dissolved in a solution of sodium triazolide, prepared from sodium hydride (55% mineral oil dispersion, 55 mg, 1.25 mmol) and triazole (97 mg, 1.4 mmol) in DMF (2.5 ml) as described above. The mixture was stirred at 65 °C for 2 h. After cooling, the mixture was diluted with AcOEt and washed with brine. Evaporation of the solvent followed by column chromatography (3 g, AcOEt) afforded **23** (117 mg, 70%) as an oil, $[\alpha]_D^{25}$ −66° (c=1.30, $CHCl_3$). MS *m/z*: 270 (M^++1), 224 (100%), 182, 156, 141, 127, 113, 82, 70. ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3H, dd, J=6.5, 1.5 Hz), 3.99 (1H, qd, J=6.5, 1 Hz), 4.57 (1H, dd, J=14, 1.5 Hz), 5.04 (1H, dd, J=14, 2 Hz), 6.5—7.0 (2H, m), 7.55 (1H, td, J=9, 7 Hz), 7.75 (1H, s), 8.12 (1H, s).

(4*R*,5*S*)-4-(2,4-Difluorophenyl)-5-methyl-4-[(1*H*-1,2,4-triazol-1-yl)-methyl]-1,3,2-dioxathiolane 2-Oxide (24) A solution of $SOCl_2$ (485 mg, 4.07 mmol) in CH_2Cl_2 (2 ml) was added dropwise to a solution of **23** (1.00 g, 3.72 mmol) and triethylamine (825 mg, 8.15 mmol) in CH_2Cl_2 (10 ml), with stirring at 0 °C. The mixture was stirred at the same temperature for 15 min and then partitioned between AcOEt and ice-water. The organic layer was washed with brine and dried. Evaporation of the solvent under reduced pressure gave an oily residue, which was chromatographed (15 g, AcOEt–benzene, 1 : 1—2 : 1, v/v) to yield **24** (1.18 g, 100%) as an oil. This product was a 5 : 4 mixture of diastereoisomers, A and B, as determined from the ^{1}H-NMR spectrum. MS *m/z*: 316 (M^++1), 251, 233, 169, 141 (100%), 127, 113, 86, 82. ^{1}H-NMR ($CDCl_3$) δ: for A; 1.87 (3H, dd, J=6.5, 1.5 Hz), 5.00 (1H, q, J=6.5 Hz), 5.05 (2H, s), 6.6—7.5 (3H, m), 7.64 (1H, s), 8.21 (1H, s): for B; 1.78 (3H, dd, J=6.5, 1.5 Hz), 4.59 (1H, d, J=14 Hz), 4.91 (1H, d, J=14 Hz), 5.20 (1H, q, J=6.5 Hz), 7.74 (1H, s), 8.05 (1H, s).

(4*R*,5*S*)-4-(2,4-Difluorophenyl)-5-methyl-4-[(1*H*-1,2,4-triazol-1-yl)-methyl]-1,3,2-dioxathiolane 2,2-Dioxide (25) A solution of **24** (900 mg, 2.85 mmol), $RuCl_3 \cdot 3H_2O$ (9 mg) and $NaIO_4$ (920 mg, 4.30 mmol) in acetonitrile (13.5 ml)–water (20 ml) was stirred at room temperature for 40 min, after which AcOEt was added and the mixture was washed with brine. Evaporation of the solvent followed by purification of the product by column chromatography (20 g, AcOEt–benzene, 1 : 1—2 : 1, v/v) afforded **25** (786 mg, 83%) as a solid. Recrystallization from benzene–hexane gave a pure specimen, mp 127—128 °C, $[\alpha]_D^{25}$ −25.9° (c=1.19, $CHCl_3$). *Anal.* Calcd for $C_{12}H_{11}F_2N_3O_4S$: C, 43.50; H, 3.35; N, 12.68. Found: C, 43.66; H, 3.38; N, 12.55. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1612, 1605, 1510. ^{1}H-NMR ($CDCl_3$) δ: 1.84 (3H, d, J=6.5, 2 Hz), 5.03 (2H, s), 5.21 (1H, q, J=6.5 Hz), 6.7—7.5 (3H, m), 7.69 (1H, s), 8.15 (1H, s).

Reaction of 25 and Sodium Azide A mixture of **25** (50 mg, 0.15 mmol), sodium azide (30 mg, 0.46 mmol) and DMF (1 ml) was stirred at 50 °C for 2 h. Then, 20% sulfuric acid (1 ml) was added and the whole was stirred at the same temperature for 4 h. After cooling, the mixture was made alkaline (pH 9) by the addition of a diluted aqueous solution of $NaHCO_3$ and concentrated under reduced pressure. Acetone was added to the residue and insoluble materials that emerged were filtered off. The filtrate was concentrated and the residue was purified by preparative TLC (AcOEt–benzene, 4 : 1, v/v) to give (2*R*,3*R*)-**3** (13 mg, 29%) as a solid. Recrystallization from benzene–hexane afforded a pure specimen, mp 139—140 °C, $[\alpha]_D^{25}$ −88° (c=0.58, $CHCl_3$), which was identical with the sample obtained above by the reaction of (2*R*,3*S*)-**2** and sodium azide.

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Tannins and Related Polyphenols of Theaceous Plants. IV.[1] Monomeric and Dimeric Hydrolyzable Tannins Having a Dilactonized Valoneoyl Group from *Schima wallichii* KORTH.

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Two new hydrolyzable tannins, schimawalins A (10) and B (17), have been isolated from the dried flowers of *Schima wallichii* (DC) KORTH. (Theaceae), and their structures, having a dilactonized valoneoyl group in the molecule, were established based on spectral and chemical evidence. Eight known hydrolyzable tannins, including camelliin B (8), the main constituent of the flower, with a macrocyclic dimer structure, were also isolated. The orientation of the valoneoyl groups in camelliin B was completely determined.

Keywords *Schima wallichii*; Theaceae; tannin; schimawalin A; schimawalin B; camelliin B; hydrolyzable tannin dimer; dilactonized valoneoyl group

Schima wallichii (DC) KORTH. (Theaceae) is widely grown in Southeast Asia, and its astringent corollas are used for the treatment of uterine disorders and hysteria, and also as an ointment to treat smallpox, in Indonesia and Malaysia.[2,3] Although this plant is known to be rich in saponins and tannins,[3] little chemical work has been reported. In a continuing study of tannins of the family Theaceae, we have examined a crude drug called "Buah cangkok" in Indonesia,[3] (dried flowers of *S. wallichii*) and isolated ten polyphenolics including camelliin B (**8**), a dimeric hydrolyzable tannin, and two new hydrolyzable tannins named schimawalins A and B.

The concentrate of 70% aqueous acetone homogenate of the flowers was rich in saponins, which cause difficulty in subsequent extraction with ether and ethyl acetate. It was treated with 20% MeOH–BuOH, and the organic layer was evaporated to dryness. The residue was passed through a column of Diaion HP-20, with water containing increasing amounts of MeOH. The eluates with 30% MeOH and 60% MeOH were further chromatographed separately to yield schimawalins A (**10**) and B (**17**), along with known tannins which were identified as 2,3-di-*O*-galloyl-D-glucose (**1**),[4] 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose (**2**),[5] gemin D (**3**),[6] tellimagrandin I (**4**) and II (**5**),[7] heterophyllin A (**6**),[8] pedunculagin (**7**)[7] and camelliin B (**8**).[9]

1 : R=OH, R'=H
2 : R=(β)-OG, R'=G

G= –CO–(3,4,5-trihydroxyphenyl)

(S)-HHDP=

3 : R=OH, R'=H, R"=G
4 : R=OH, R'=R"=G
5 : R=(β)-OG, R'=R"=G
6 : R=(α)-OG, R'=H, R"=G
7 : R=OH, R'~R"=(S)-HHDP

Chart 1

Camelliin B (**8**), which is the main tannin of this crude drug, was previously isolated from the flower buds of *Camellia japonica* L. and *C. sasanqua* THUNB., and its unique macrocyclic structure, except for the orientation of the valoneoyl group at O-4'/O-6', was established as **8**.[9] The orientation of the valoneoyl group has been

glucose-I

glucose-II

8

9

Chart 2

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determined in the present study as follows. Reduction of **8** with $NaBH_4$ furnished a dihydro derivative (**9**), which shows an $(M+Na)^+$ ion peak at *m/z* 1745 in the fast-atom bombardment mass spectrum (FAB-MS). Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a fairly well-resolved sharp peak for each proton, unlike the complex spectrum of **8**, which forms an equilibrium mixture of α- and β-anomers. The ^{1}H–^{1}H shift correlation spectrum (COSY) of **9** indicated that the glucose-II of **8** was reduced to a glucitol (see Experimental).

Among the valoneoyl proton signals (δ 6.18, 6.32, 6.64, 6.79, 6.83, 7.19) in the ^{1}H-NMR spectrum of **9**, the H_A ($H_{A'}$) and H_B ($H_{B'}$) signals (δ 6.18—6.79) of each valoneoyl group were distinguished from the H_C ($H_{C'}$) signal [δ 7.19 (6.83)], by the correlations of the former two signals with C-1 and C-1′ at δ 115.7—117.3 through three-bond couplings in the ^{1}H–^{13}C long-range COSY. The signals at δ 6.18 and 6.32 were assigned to H_B ($H_{B'}$), based on the correlation through two-bond coupling with the signals at δ 146.2 and 146.3, which are attributable to the phenyl ether carbons (C-4′). The remaining two singlets at δ 6.79 and 6.64 were thus attributed to H_A ($H_{A'}$). A connectivity between H-4′ [δ 5.33 (dd, J=3.0, 9.0 Hz)] of the glucitol core and valoneoyl H_A (δ 6.79) was indicated by the three-bond correlations with the carbonyl carbon signal at δ 168.8 (Fig. 1). Similarly, the glucitol H-6′ signal [δ 4.55 (dd, J=5.0, 12.5 Hz)] was correlated with the carbonyl signal at δ 169.4, which showed a cross peak with the valoneoyl H_B at δ 6.18. The orientation of the valoneoyl group at O-4′/O-6′ in **9**, and consequently in camellin B (**8**), was thus established. The other long-range correlations through three-bond couplings illustrated in the formula **9**, also provided further evidence for the proposed structure (**8**) of camelliin B.

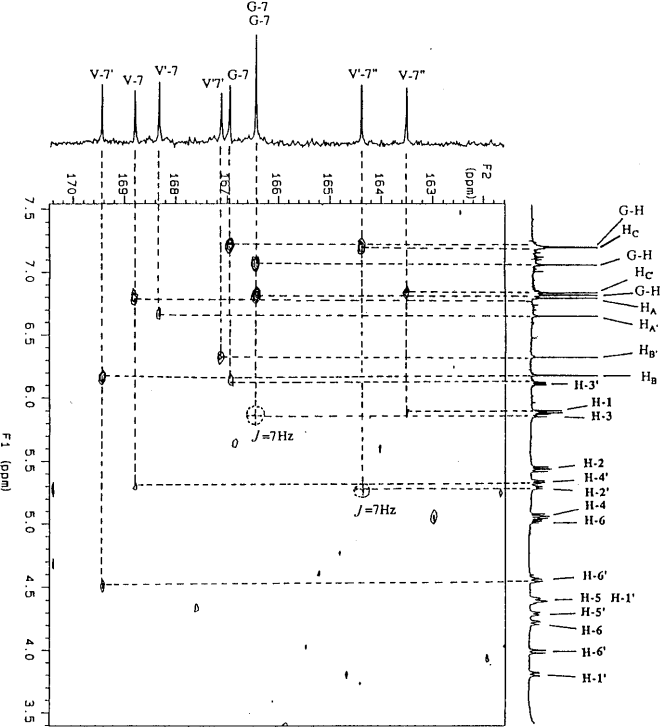

Fig. 1. A Part of ^{1}H–^{13}C Long Range Shift Correlation Spectrum of Dihydrocamelliin B (**9**) (Acetone-d_6 + D_2O, J_{CH} = 8 Hz)

Cross peaks in dotted circles were observed in a separate experiment with an average J_{CH} value of 7 Hz.

A new tannin, schimawalin A (**10**), was obtained as a light brown amorphous powder, $[\alpha]_D$ +74.3° (MeOH). Its FAB-MS exhibited the $(M+Na)^+$ ion peak at *m/z* 1277, which is consistent with the molecular formula $C_{55}H_{34}O_{35}$. Schimawalin A (**10**) forms an equilibrium mixture of α- and β-anomers as revealed by duplication of each signal in the ^{1}H-NMR spectrum. The presence of a dilactonized valoneoyl group in **10** was indicated by the characteristic low field aromatic signals[10] [δ 7.60, 7.59 (1H in total), 7.12, 7.11 (1H in total), 7.09, 7.08 (1H in total)], and by the ultraviolet (UV) absorption at 360 nm.[11]

10

11 : R~R′=(S)-HHDP
12 : R=R′=H

14

15

16

13

Chart 3

The paired signals were also exhibited for a galloyl group and a valoneoyl group (see Experimental). The coupling patterns of the aliphatic proton signals which were assigned by ^{1}H–^{1}H COSY are typical of the $^4C_1$ glucopyranose residue with a free anomeric hydroxyl group. The *S*-configuration of the valoneoyl group in **10** was indicated by a positive Cotton effect at 223 nm[12] in the circular dichroism (CD) spectrum. Schimawalin A is therefore regarded as an ellagitannin composed of a galloyl, and (*S*)-valoneoyl, a dilactonized valoneoyl group and glucose. These structural characteristics are similar to those of cornusiin B (**11**),[13] and the sugar proton signals of **10** are virtually identical with those of **11**, as shown in Table I. A feature distinguishing **10** and **11** is the difference of the chemical shifts of the H-6 signals, which indicates that the valoneoyl group of **10** is at O-4/O-6, and that the other acyl groups on the glucose residue are located similarly to those of **11**. The orientation of the valoneoyl group at O-4/O-6, as in the structure **10**, was determined based on the analogy of the chemical shifts of the valoneoyl H_A signal (δ 6.46, 6.42) to those (δ 6.46, 6.44) of rugosin B (**13**),[14] whose structure was already established by ^{1}H–^{13}C long-range COSY.

Table I. ^{1}H-NMR Data for the Glucose Moieties of **10** and **11** (500 MHz, Acetone-d_6 + D_2O, J in Hz)

	10		11[a]	
	α-Anomer	β-Anomer	α-Anomer	β-Anomer
H-1	5.32 d (J=3.8)	4.70 d (J=8.0)	5.32 d (J=3.5)	4.70 d (J=8.0)
H-2	5.04 dd (J=3.8, 10.0)	5.15 dd (J=8.0, 10.0)	5.05 dd (J=3.5, 10.0)	5.15 dd (J=8.0, 10.0)
H-3	5.70 t (J=10.0)	5.30 t (J=10.0)	5.76 t (J=10.0)	5.38 t (J=10.0)
H-4	4.95 t (J=10.0)	4.89 t (J=10.0)	5.01 t (J=10.0)	4.97 t (J=10.0)
H-5	4.52 m	4.00 m	4.56 br dd (J=6.5, 10.0)	4.06 br dd (J=6.5, 10.0)
H-6	5.09 dd (J=6.0, 13.0)	5.09 dd (J=6.0, 13.0)	5.21 dd (J=6.5, 12.5)	5.22 dd (J=6.5, 13.0)
	3.57 d (J=13.0)	3.57 d (J=13.0)	3.66 dd (J=1.0, 12.5)	3.71 dd (J=0.5, 13.0)

a) Data are taken from ref. 13.

The chemical evidence for the structure (**10**) of schimawalin A was obtained as follows. Upon treatment with hot water containing a small amount of trifluoroacetic acid, **10** gave gallic acid (**14**), ellagic acid (**15**), valoneic acid dilactone (**16**) and oenothein C (**12**).[13] A milder partial hydrolysis of **10** with hot water furnished **14** and cornusiin B (**11**). Among these products, ellagic acid (**15**) and cornusiin B (**11**) are regarded as having been formed by the cleavage of an ether bond of the valoneoyl group of **10**, in an analogous way to the partial hydrolysis of a variety of tannins possessing a valoneoyl group in the molecule.[15] Based on these findings, the structure **10** was assigned to schimawalin A.

Shimawalin B (**17**) is a dimeric hydrolyzable tannin as indicated by the $(M+Na)^+$ ion peak at m/z 1743 in FAB-MS. The duplication of each signal in the ^{1}H-NMR spectrum implied that **17** forms an equilibrium mixture of two anomers, like **10**. The presence of three galloyl groups in **17** was indicated by three pairs of signals [δ 7.03, 7.06 (2H in total), 6.89, 6.89 (2H in total) and 6.73, 6.75 (2H in total)] in the aromatic region. The presence of two valoneoyl groups, one of which is dilactonized, was also shown by six aromatic 1H singlets appearing as paired signals similar to those of **10**. The anomeric proton signals [δ 6.08 (1H, d, J=8.0 Hz, H-1), 5.18 (1/4H, J=3.0 Hz, H-1′) and 4.22 (3/4H, d, J=8.0 Hz, H-1′)] and other sugar proton signals, of large coupling constants (J=8—13.5 Hz), indicate the presence of two $^4C_1$ glucopyranose residues, one of which has a free hydroxyl group at the anomeric center. The chemical shift of H-1′ (δ 4.23) of the β-anomer, which is shifted to higher field than that (δ 5.13) of **4** and is comparable to those (δ 4.70—4.22) of **8** and **10**—**12**, indicates that the galloyl part of the valoneoyl group is located at O-2′. The presence of free hydroxyl groups at O-4′ and O-6′ of the glucose-II was also indicated by the H-4′—H-6′ signals in the upfield region (δ 3.6—3.9). The chemical shifts and coupling pattern of the glucose-I residue are virtually identical with those of prostratin A (**18**), which was recently isolated from *Euphorbia prostrata*,[10] suggesting an analogy of the substitution pattern on the

19

17 **18**

Chart 4

glucose core to that in **18**. The valoneoyl group in **17** is thus located at O-4/O-6 of the glucose-I, and its orientation is indicated to be the same as that of isorugosin B (**19**)[16] by the chemical shift of the H_A signal (δ 6.62), which is similar to that (δ 6.65) of **19**. In fact, **19** and 2,3-di-*O*-galloylglucose (**1**) were formed upon partial hydrolysis of **17** with hot water. Schimawalin B is, therefore, assigned the structure **17**. Although this dimer (**17**) is one of the degradation products obtained from camelliin B,[9] this is the first report of its isolation from a natural source.[17]

Experimental

General Instruments ([α]$_D$, UV, NMR, MS) used in this work were the same as those described in the preceding paper.[1] Reversed-phase high-performance liquid chromatography (HPLC) was conducted on a column of LiChrospher RP-18 (5 μm) (4 × 250 mm) using 0.01 M H_3PO_4–0.01 M KH_2PO_4–EtOH–EtOAc (42.5 : 42.5 : 10 : 5), in an oven at 40 °C. Normal-phase HPLC was carried out on a column of Superspher Si 60 (4 × 119 mm) developed with hexane–MeOH–tetrahydrofuran–HCOOH (60 : 45 : 15 : 1) containing oxalic acid (500 mg/l). Toyopearl HW-40 (Tosoh, Corp.), Diaion HP-20 and MCl-Gel CHP-20P (Mitsubishi Kasei Co., Ltd.) were used for column chromatography.

Plant Material The dried flowers of *S. wallichii* (DC) KORTH. (Indonesian trivial name, "buah cangkok") were purchased at a market in Bogor, Indonesia, in September 1989, and a voucher specimen (AN-BJ No. 84) has been deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Kyoto University.

Isolation of Tannins The dried flowers (940 g) were homogenized in 70% acetone (10 l) and filtered. The filtrate was concentrated to *ca.* 1.5 l. The sticky precipitate was filtered off, and the filtrate was extracted with *n*-BuOH–MeOH (8 : 2). The organic layer was evaporated to give a dark brown residue (63 g), and a part (30 g) of this residue was suspended in H_2O. After removal of insoluble materials (15 g) by filtration, the water-soluble portion was subjected to column chromatography over Diaion HP-20 (3.5 cm i.d. × 30 cm) with H_2O→20% MeOH→30% MeOH→40% MeOH→60% MeOH→MeOH. The 30% MeOH and 40% MeOH eluates were combined and rechromatographed over Toyopearl HW-40 (fine grade) [50% MeOH→60% MeOH→70% MeOH→MeOH→acetone–H_2O (7 : 2 : 1)] to yield 2,3-di-*O*-galloylglucose (**1**) (13 mg), gemin D (**3**) (8 mg), pedunculagin (**7**) (7 mg), tellimagrandin I (**4**) (24 mg) and camelliin B (**8**) (2 mg). The 60% MeOH eluate (880 mg) was also chromatographed on Toyopearl HW-40 (fine grade) using the same solvent system to give 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**2**) (14 mg), crude schimawalin A (30 mg), tellimagrandin I (4) (33 mg), heterophyllin A (**6**) (7 mg), tellimagrandin II (**5**) (29 mg), crude schimawalin B (25 mg) and camelliin B (66 mg). Crude schimawalins A (**10**) and B (**17**) were separately purified by column chromatography over MCl-gel CHP-20P (H_2O→20% MeOH→30% MeOH to give **10** (1.5 mg) and **17** (4.5 mg).

Reduction of Camelliin B A mixture of **8** (100 mg) and $NaBH_4$ (100 mg) in H_2O (20 ml) was left standing at room temperature for 30 min. After acidification with diluted HCl (pH 3), the reaction mixture was directly applied on a column of MCl-Gel CHP-20P (1.1 cm i.d. × 25 cm) and eluted with H_2O containing increasing amounts of MeOH in a stepwise gradient mode. The 30% MeOH eluate gave dihydrocamelliin B (**9**) (27 mg) as a light yellow amorphous powder, [α]$_D$ +52.5° (*c* = 1.0, MeOH). FAB-MS *m/z*: 1745 $(M+Na)^+$. *Anal.* Calcd for $C_{75}H_{54}O_{48}$·10H_2O: C, 47.32; H, 3.89. Found: C, 47.26; H, 4.18. UV λ_{max} (MeOH) nm (log ε): 217 (5.01), 272 (4.61). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.20, 7.06, 6.81 [each 2H, s, galloyl (G)], 5.89 (1H, d, *J* = 8.0 Hz, H-1), 5.44 (1H, dd, *J* = 8.0, 10.0 Hz, H-2), 5.87 (1H, t, *J* = 10.0 Hz, H-3), 5.06 (1H, t, *J* = 10.0 Hz, H-4), 4.39 (1H, m, H-5), 5.02 (1H, dd, *J* = 8.6, 12.5 Hz, H-6), 4.20 (1H, dd, *J* = 2.5, 12.5 Hz, H-6), 3.80 (1H, dd, *J* = 4.0, 13 Hz, H-1′), 4.40 (2H, m, H-1′ and H-5′), 5.28 (1H, m, H-2′), 6.11 (1H, dd, *J* = 3.0, 9.0 Hz, H-3′), 5.33 (1H, dd, *J* = 3.0, 9.0 Hz, H-4′), 4.28 (1H, dd, *J* = 3.0, 9.0 Hz, H-5′), 4.55 (1H, dd, *J* = 5.0, 12.5 Hz, H-6′), 3.98 (1H, d, *J* = 12.5 Hz, H-6′), valoneoyl and dilactonized valoneoyl protons, see text. ^{13}C-NMR (acetone-d_6+D_2O) δ: 94.1 (C-1), 71.1 (C-2), 74.2 (C-3), 71.7 (C-4), 72.3 (C-5), 63.8 (C-6), 61.5 (C-1′), 75.3 (C-2′), 71.1 (C-3′), 73.7 (C-4′), 68.6 (C-5′), 68.9 (C-6′), 104.8, 106.1 [valoneoyl (Val) C-3′], 108.3, 108.5 (Val C-3), 108.9, 109.4 (Val C-6″), 110.0, 110.1, 110.4 [each 2C, galloyl (G) C-2, 6], 113.2, 115.9 (Val C-1″), 115.7, 116.3, 117.0, 117.3 (Val C-1, C-1′), 120.0, 120.2, 120.5 (G C-1), 125.0, 125.1, 125.7, 126.6 (Val C-2, C-2′), 136.2, 136.9 (Val C-5′), 136.6, 136.5 (Val C-5), 137.3, 137.8 (Val C-2″), 139.0, 139.3, 139.4 (G C-4), 139.4, 139.6 (Val C-3″), 140.4, 140.6 (Val C-4″), 142.0, 143.0 (Val C-5″), 144.5, 144.6, 144.7, 145.0, 145.1 (Val C-6, C-6′, C-4), 145.6, 145.7, 145.9 (G C-3, C-5), 146.2, 146.3 (Val C-4′), 163.5, 164.4 (Val C-7″), 166.5 (2C) (G C-7), 167.0 (G C-7), 167.1, 168.4, 168.8, 169.4 (Val C-7, 7′).

Schimawalin A (10) An off-white amorphous powder, [α]$_D$ +74.3° (*c* = 0.4, MeOH). FAB-MS *m/z*: 1277 $(M+Na)^+$. UV λ_{max} (MeOH) nm (log ε): 206 (4.95), 258 (4.56), 360 (3.70). CD (MeOH) [θ] (nm): $+5.6 \times 10^4$ (223), -1.3×10^4 (261), $+2.0 \times 10^4$ (287). ^{1}H-NMR (acetone-d_6+D_2O) δ: 6.82, 6.73 [each s, 2H in total, galloyl (G)], 7.60, 7.59 (each s, 1H in total), 7.12, 7.11 (each s, 1H in total), 7.09, 7.08 (each s, 1H in total)· [dilactonized valoneoyl (DLV)], 7.07, 7.00 (each s, 1H in total), 6.46, 6.42 (each 1H in total), 6.19, 6.18 (each s, 1H in total) (Val), glucose protons, see Table I.

Partial Hydrolysis of Schimawalin A (10) a) A solution of **10** (0.5 mg) in H_2O containing CF_3COOH (1 drop) was heated in a boiling-water bath for 30 min, and the reaction mixture was extracted with AcOEt. HPLC (reversed-phase) analysis of the AcOEt extract indicated the formation of gallic acid (**14**) (t_R 2.92 min), valoneic acid dilactone (**16**) (t_R 7.65 min) and oenothein C (**12**) (t_R 3.39 min). The identities of these products were confirmed by co-chromatography with authentic samples.

b) An aqueous solution (0.5 ml) of **10** (0.5 mg) was heated in a boiling-water bath for 30 min. The reaction mixture was directly analyzed by HPLC (reversed-phase) to detect gallic acid and cornusiin B (**11**) (t_R 4.65 and 5.70 min).

Schimawalin B (17) An off-white amorphous powder, [α]$_D$ +75° (*c* = 1.0, MeOH). FAB-MS: *m/z* 1743 $(M+Na)^+$. *Anal.* Calcd for $C_{75}H_{52}O_{48}$·4H_2O: C, 50.28; H, 3.35. Found: C, 50.26; H, 3.63. UV λ_{max} (MeOH) nm (log ε): 208 (4.66), 270 (4.57), 358 (3.61). CD (MeOH) [θ] (nm): $+13.8 \times 10^4$ (220), $+6.8 \times 10^4$ (237), -3.1×10^4 (257), $+4.0 \times 10^4$ (283). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.03, 7.06 (each s, 2H in total), 6.89 (2H, s), 6.73, 6.75 (each s, 2H in total) (G), 7.59 (1H, s), 7.18 (1H, s), 7.10 (1H, s) (DLV), 7.00 (1H, s), 6.61, 6.62 (each s, 1H in total), 6.10, 6.13 (each s, 1H in total) (Val), 6.08 [d, *J* = 8.0 Hz, H-1 of α-anomer and β-anomer (α and β)], 5.49 [dd, *J* = 8.0, 10.0 Hz, H-2 (α)], 5.45 [d, *J* = 8.0, 10.0 Hz, H-2 (β)], 5.53 [t, *J* = 10.0 Hz, H-3 (α)], 5.50 [t, *J* = 10.0 Hz, H-3 (β)], 4.95 (t, *J* = 10.0 Hz, H-4 (α)], 5.00 [t, *J* = 10.0 Hz, H-4 (β)], 4.34, 4.36 [m, H-5 (α and β)], 5.14 [dd, *J* = 6.0, 13.5 Hz, H-6 (α)], 5.20 [dd, *J* = 6.0, 13.5 Hz, H-6 (β)], 3.70 [d, *J* = 13.5 Hz, H-6 (α and β)], 5.18 [d, *J* = 3.0 Hz, H-1′ (α)], 4.87 [dd, *J* = 3.0, 10.0 Hz, H-2′ (α)], 5.29 [t, *J* = 10.0 Hz, H-3′ (α)], 3.81 [t, *J* = 10.0 Hz, H-4′ (α)], 4.22 [d, *J* = 8.0 Hz, H-1′ (β)], 4.89 [dd, *J* = 8.0, 10.0 Hz, H-′2 (β)], 5.27 [t, *J* = 10.0 Hz, H-3′ (β)], 3.59 [t, *J* = 10.0 Hz, H-4′ (β)], 3.9—3.7 (H-5′, 6′ (α and β)].

Partial Hydrolysis of Schimawalin B (17) An aqueous solution (1 ml) of **17** (1 mg) was heated in a boiling-water bath for 3 h. The HPLC (reversed-phase) analysis of the reaction mixture showed the production of **19** (t_R 3.57 and 4.87 min), 2,3-di-*O*-galloylglucose (**1**) (t_R 2.55 and 2.76 min) and valoneic acid dilactone (**16**) (t_R 7.65 min).

Acknowledgements The authors are grateful to Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University, for CD measurements, and to the SC-NMR Laboratory of Okayama University for NMR experiments using VXR 500 instument. One of the authors (T. C.) is grateful to the Ministry of Education, Science and Culture of Japan for a scholarship. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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17) The possibility that schimawalin B may be an artefact derived from camelliin B can not be ruled out at present.

New Coumarins from *Citrus hassaku*[1)]

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Five new coumarins named nordenletin (1), oxanordentatin (3), oxaclausarin (4), seselinol (6), and seselinol isovalerate (8) were isolated from the roots of *Citrus hassaku* (Rutaceae) and their structures were elucidated on the basis of spectrometric analyses.

Keywords *Citrus hassaku*; Rutaceae; coumarin; nordenletin; oxanordentatin; oxaclausarin; seselinol; seselinol isovalerate

During our investigations on the constituents of *Citrus* plants (Rutaceae), we have reported the isolation of many kinds of new coumarins and acridone alkaloids.[2)] As a part of this program, we have studied the constituents of the roots of *Citrus hassaku* HORT. *ex* TANAKA and isolated many kinds of coumarins and acridone alkaloids, including some new compounds. In this paper, we wish to report the isolation and structure elucidations of five new coumarins.

Results and Discussion

Structure of Nordenletin (1) Nordenletin (**1**) was isolated as light yellow prisms, mp 265—270 °C, $C_{33}H_{32}O_8$, $[\alpha]_D$ $-61.8°$ (pyridine). The presence of a 5,7-dioxygenated coumarin nucleus was suggested by the ultraviolet (UV) [λ_{max} 214, 267, 286, 321 (sh) and 343 nm] and infrared (IR) (3300, 1720, 1620 cm^{-1}) spectra.[3)] The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of (**1**) showed signals due to two hydroxyl groups (δ 10.04 and 9.43), and two pairs of characteristic signals due to H-4, H-3 and H-4′, H-3′ of two coumarin nuclei [δ 8.18, 6.19 (each 1H, d, J=9.52 Hz) and 7.99, 6.00 (each 1H, d, J=9.52 Hz)]. From the chemical shifts of both H-4 and H-4′, C-5 and C-5′ of both coumarin nuclei were assumed to be *O*-substituted.[4)]

Furthermore, signals assignable to a 1,1-dimethylpyran ring [δ 6.64, 5.56 (each 1H, d, J=10.26 Hz), 1.25 and 0.57 (each 3H, s)] and a 1,1-dimethylallyl group [δ 6.28 (1H, dd, J=17.58, 10.62 Hz), 4.89 (1H, d, J=17.58 Hz), 4.81 (1H, d, J=10.62 Hz), 1.65 and 1.63 (each 3H, s)] were observed. The remaining signals at δ 4.69 (1H, dd, J=8.4, 11.2 Hz), 2.05 (1H, dd, J=13.9, 11.2 Hz), 1.95 (1H, dd, J=13.9, 8.4 Hz), and 1.26 and 1.42 (each 3H, s) suggested the presence of substituents at C-11′ of the dihydro 1,1-dimethylpyran ring. Though the molecular ion peak could not be detected in the electron impact-mass spectrum (EI-MS), two fragments arising from the benzylic fission were observed at m/z 312 and 244. The fast atom bombardment-mass spectrum (FAB-MS) showed $M^+ + 1$ at m/z 557 which corresponds to $C_{33}H_{32}O_8$. The above data suggested the binary pyranocoumarin structure of this compound. Nordenletin (**1**) was methylated by diazomethane to afford the dimethoxy compound (**2**). The molecular formula $C_{35}H_{36}O_8$ was established by high-resolution-MS (HR-MS) and it revealed the binary structure of (**1**). The types of two linked coumarin (linear or angular type) and the binding positions of the two coumarin nuclei were determined by nuclear Overhauser effect (NOE) experiments with **2**. Irradiation of the methoxy

TABLE I. ^{1}H- and ^{13}C-NMR Data for Nordenletin (**1**) in DMSO-d_6

Atom	^{13}C	^{1}H	Atom	^{13}C	^{1}H
2	160.36 (s)		2′	159.91 (s)	
3	109.37 (d)	6.19 (1H, d, 9.52)	3′	108.29 (d)	6.00 (1H, d, 9.52)
4	140.19 (d)	8.18 (1H, d, 9.52)	4′	139.89 (d)	7.99 (1H, d, 9.52)
4a	103.15 (s)		4a′	103.15 (s)	
5	147.74 (s)		5′	150.64 (s)	
6	105.75 (s)		6′	110.61 (s)	
7	153.75 (s)		7′	157.66 (s)	
8	111.99 (s)		8′	113.29 (s)	
8a	152.64 (s)		8a′	151.81 (s)	
9	75.87 (s)		9′	75.70 (s)	
9-Me	25.84 (q)	0.57 (3H, s)	9′-Me	22.23 (q)	1.26 (3H, s)
	27.42 (q)	1.25 (3H, s)		29.14 (q)	1.42 (3H, s)
10	127.93 (d)	5.56 (1H, d, 10.26)	10′	37.60 (t)	1.95 (1H, dd, 13.9, 8.4)
11	115.80 (d)	6.64 (1H, d, 10.26)			2.05 (1H, dd, 13.9, 11.2)
Others		10.04 (OH)	11′	24.69 (d)	4.69 (1H, dd, 11.2, 8.4)
		9.43 (OH)	1″	40.17 (s)	
			1″-Me	29.21 (q)	1.63 (3H, s)
				30.06 (q)	1.65 (3H, s)
			2″	150.22 (d)	6.28 (1H, dd, 17.58, 10.62)
			3″	107.18 (t)	4.81 (1H, d, 10.62)
					4.89 (1H, d, 17.58)

Values are in ppm. The coupling constants (J values) in parentheses are in Hz.

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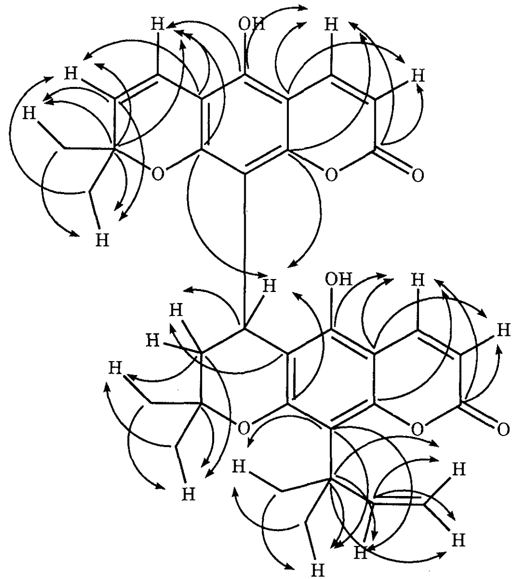

Fig. 1. CH Long-Range Correlations in the HMBC Spectrum (J=8 Hz) of Nordenletin (**1**)

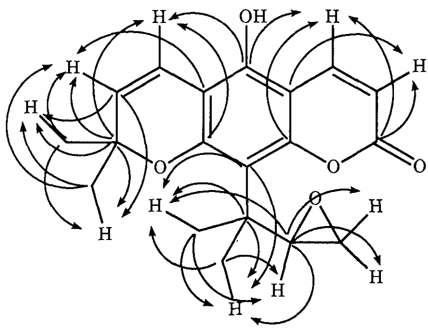

Fig. 2. CH Long-Range Correlations in the HMBC Spectrum (J=8 Hz) of Oxanordentatin (**3**)

signal at δ 3.84 enhanced the signals at δ 7.95 (H-4) and 6.47 (H-11), while irradiation of the signal at δ 3.61 enhanced the signals at δ 7.67 (H-4') and 4.93 (H-11') about 7—8%, respectively. These results suggested the structure of *O,O'*-dimethylnordenletin to be **2**. The structure of nordenletin was further confirmed by ^{1}H–^{13}C shift correlation spectroscopy (^{1}H–^{13}C COSY) and ^{1}H-detected multiple-bond multiple-quantum coherence (HMBC) spectroscopy of **1**. The assignments of carbons and protons (Table I) were made on the basis of the ^{1}H–^{13}C COSY and HMBC results shown by arrows in Fig. 1. The presence of two linear pyranocoumarin skeletons was confirmed by cross peaks of C-5 (δ 147.74) to H-4 (δ 8.18) and H-11 (δ 6.64); C-5' (δ 150.64) to H-4' (δ 7.99); C-6' (δ 110.61) and C-11' (δ 24.69) to H-10' (δ 1.95). The presence of the 1,1-dimethylallyl group at C-8' was indicated by the cross peak of C-8' (δ 113.29) to H-2'' (δ 6.28). Two phenolic hydroxyl groups were determined to be located at C-5 and C-5' from the chemical shifts of these carbons. The linking position of the two coumarin nuclei at C-8 and C-11' was confirmed by cross peaks of C-7 (δ 153.75), C-8a (δ 152.64) and C-7' (δ 157.66) to H-11' (δ 4.69). From the aforementioned results, the structure of nordenletin was concluded to be **1**.

Structure of Oxanordentatin (3) Oxanordentatin (**3**) was isolated as a colorless oil, $[\alpha]_D$ $-59.0°$ (EtOH). The molecular formula $C_{19}H_{20}O_5$ was established by HR-MS. The UV [λ_{max} 224, 269 (sh), 293 and 338 nm] and IR (3580, 1720, 1605 cm^{-1}) spectra[3] suggested the 5,7-dioxygenated coumarin structure of **3**. The ^{1}H-NMR spectrum showed characteristic signals of H-4 and H-3 [δ 7.94 and 6.10 (each 1H, d, J=9.75 Hz)] and the presence of a 1,1-dimethylpyran ring [δ 6.48, 5.57 (each 1H, d, J=9.75 Hz) and 1.47 (6H, s)]. From these data, this coumarin was considered to be a pyranocoumarin. Furthermore, as signals of the side chain, a methylene [δ 3.93 (2H, m)], methine [δ 4.50 (1H, dd, J=4.04, 7.73 Hz)] and two methyl proton signals [δ 1.59 and 1.37 (each 3H, s)] were observed. The orientation of the pyran ring and the location of the side chain on the coumarin skeleton were determined from the ^{1}H–^{13}C COSY and HMBC spectra (Fig. 2). The cross peaks of C-5 (δ 150.46) to H-4 (δ 7.94) and H-11 (δ 6.48) suggested the linear structure and the chemical shift of C-5 together with the UV spectrum also suggested the presence of a hydroxy group at this position. The epoxide structure of the 1,1-dimethylallyl substituent was deduced from the cross peaks of C-2' (δ 94.57) to H-3' (δ 3.93); methyl carbons (δ 20.91 and 26.95) to H-2' (δ 4.50); C-1' (δ 43.42) and C-2' (δ 94.57) to methyl protons (δ 1.59 and 1.37). The location of this alkyl group was also elucidated by the cross peak of C-8 (δ 113.72) to methyl protons (δ 1.59 and 1.37). From the above results, the structure of oxanordentatin was concluded to be **3**.

Structure of Oxaclausarin (4) Oxaclausarin (**4**) was isolated as a colorless oil, $[\alpha]_D$ $-32.3°$ (EtOH). The molecular formula $C_{24}H_{28}O_5$ was determined from its MS. The 5,7-dioxygenated coumarin skeleton was also suggested from its UV [λ_{max}: 226, 272 (sh), 284, 304 (sh) and 338 nm] and IR (3580, 1715, 1603 cm^{-1}) spectra.[3] In the ^{1}H-NMR spectrum of **4**, characteristic signals of H-4 of a coumarin skeleton [δ 7.79 (s)], a 1,1-dimethylpyran ring [δ 6.48 (1H, d, J=10.01 Hz), 5.55 (1H, d, J=10.01 Hz) and 1.47 (6H, s)], a 1,1-dimethylallyl group [δ 6.19 (1H, dd, J=10.5, 17.58 Hz), 5.09 (1H, dd, J=1.22, 17.58 Hz), 5.07 (1H, dd, J=1.22, 10.5 Hz) and 1.47 (6H, s)], methylene and methine protons [δ 3.90 (2H, m) and 4.48 (1H, dd, J=7.57, 3.91 Hz)] and two methyl groups [δ 1.58 and 1.35 (each 3H, s)] were observed. These data resembled those of clausarin (**5**)[5] and oxanordentatin (**3**). Comparisons of ^{1}H- and ^{13}C-NMR spectral data (Tables II and III) suggested that one of the 1,1-dimethylallyl groups of clausarin (**5**) was oxidized in oxaclausarin (**4**). The determination of the location of the oxidized substituent was performed by examination of the ^{1}H–^{13}C COSY and HMBC spectra (Fig. 3). The cross peaks of C-1'' (δ 40.40) to H-4 (δ 7.79) and H-3'' (δ 5.09 and 5.07); C-3 (δ 129.09) to methyl protons (δ 1.47) suggested the location of the 1,1-dimethylallyl group to be at C-3. On the other hand, the cross peaks of C-8 (δ 112.89) and C-2' (δ 94.43) to methyl protons at C-1' (δ 1.58 and 1.35); C-2' (δ 94.43) to H-3' (δ 3.90); methyl carbons at C-1' (δ 20.95, 27.01) to H-2' (δ 4.48) suggested the location of the epoxidized substituent at C-8. From these data, the structure of oxaclausarin was concluded to be **4**.

Structure of Seselinol (6) Seselinol (**6**) was isolated as a light yellow oil, $[\alpha]_D$ $-111.6°$ ($CHCl_3$). The molecular formula $C_{14}H_{12}O_4$ was obtained by HR-MS and the 7-oxygenated coumarin nucleus was suggested by its IR

1 : R = H
2 : R = Me

3 : R = H
4 : R = 1″,2″,3″ (1,1-dimethylallyl)

5

6 : R = OH
7 : R = H
8 : R = $OCOCH_2CH(CH_3)_2$

Chart 1

TABLE II. ^{1}H-NMR Data for Oxanordentatin (3), Oxaclausarin (4), and Clausarin (5)

Compd.	3	4	5
H-3	6.10 (d, J=9.75)		
H-4	7.94 (d, J=9.75)	7.79 (s)	7.85 (s)
9-Me	1.47 (6H, s)	1.47 (6H, s)	1.47[a] (6H, s)
H-10	5.57 (d, J=9.75)	5.55 (d, J=10.01)	5.67 (d, J=9.77)
H-11	6.48 (d, J=9.75)	6.48 (d, J=10.01)	6.50 (d, J=9.77)
1′-Me	1.59 (3H, s), 1.37 (3H, s)	1.58 (3H, s) 1.35 (3H, s)	1.63 (6H, s)
H-2′	4.50 (dd, J=4.04, 7.73)	4.48 (dd, J=3.91, 7.57)	6.29 (dd, J=10.5, 17.58)
H-3′	3.93 (2H, m)	3.90 (2H, m)	4.93 (dd, J=1.22, 17.58) 4.85 (dd, J=1.22, 10.5)
1″-Me		1.47 (6H, s)	1.43[a] (6H, s)
H-2″		6.19 (dd, J=10.5, 17.58)	6.18 (dd, J=10.5, 17.58)
H-3″		5.09 (dd, J=1.22, 17.58) 5.07 (dd, J=1.22, 10.5)	5.11 (dd, J=0.97, 17.58) 5.07 (dd, J=0.97, 10.5)

Values are in ppm. The coupling constants (J values) in parentheses are in Hz. a) Assignments may be interchanged.

(3450, 1730, 1610 cm^{-1}) and UV [λ_{max} 218, 283 (sh), 294 and 330 nm] spectra.[3] The ^{1}H-NMR spectrum showed three pairs of doublets at δ 7.60, 6.24 (each 1H, d, J=9.21 Hz), 7.23, 6.75 (each 1H, d, J=8.74 Hz) and 7.05, 5.71 (each 1H, d, J=10.08 Hz), characteristic signals of H-4, H-3, H-5, H-6 of a coumarin skeleton and H-11, H-10 of a dihydropyran ring, respectively. The remaining signals at δ 6.72 (1H, s), 3.70 (2H, br s) and 1.43 (3H, s) suggested the presence of hydroxymethyl and methyl groups. The comparisons of these data with those of seselin (7)[6] indicated the presence of the hydroxymethyl group in place of one of the geminal dimethyl groups (Table IV). From the aforementioned results, the structure of seselinol was concluded to be **6**.

TABLE III. ^{13}C-NMR Data for Compounds 3, 4, and 5

Compound	3	4	5
C-2	161.01	159.62	160.8
C-3	110.16	129.09	128.53
C-4	139.19	132.96	134.18
C-4a	103.93	104.05	104.44
C-5	150.46	150.11	147.04
C-6	101.96	103.75	106.38
C-7	157.42	156.49	155.11
C-8	113.72	112.89	115.20
C-8a	150.94	150.18	153.20
C-9	78.01	77.82	79.0
9-Me	27.99, 28.08	27.98, 28.08	27.27 (×2)
C-10	128.05	127.76	129.28
C-11	115.71	115.90	115.84
C-1′	43.42	43.40	40.88
1′-Me	20.91, 26.95	20.95, 27.01	29.50 (×2)
C-2′	94.57	94.43	150.12
C-3′	61.69	61.75	107.94
C-1″		40.40	40.18
1″-Me		26.28 (×2)	26.19 (×2)
C-2″		145.74	145.63
C-3″		111.77	111.90

Values are in ppm.

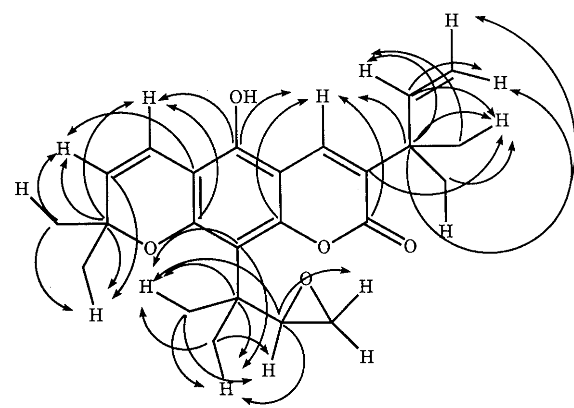

Fig. 3. CH Long-Range Correlations in the HMBC Spectrum (J=8 Hz) of Oxaclausarin (4)

TABLE IV. ^{1}H-NMR Data for Seselinol (6), Seselin (7), and Seselinol Isovalerate (8)

Compd.	6	7	8
H-3	6.24 (d, J=9.21)	6.22 (d, J=9.52)	6.24 (d, J=9.52)
H-4	7.60 (d, J=9.21)	7.59 (d, J=9.52)	7.60 (d, J=9.52)
H-5	7.23 (d, J=8.74)	7.21 (d, J=8.55)	7.23 (d, J=8.55)
H-6	6.75 (d, J=8.74)	6.72 (dd, J=0.73, 8.55)	6.72 (dd, J=8.55, 0.74)
H-10	5.71 (d, J=10.08)	5.73 (d, J=10.01)	5.66 (d, J=10.25)
H-11	7.05 (d, J=10.08)	6.88 (dd, J=0.73, 10.01)	7.03 (dd, J=10.25, 0.74)
9-Me	1.43 (3H, s)	1.48 (6H, s)	1.49 (3H, s)
Other signals	6.72 (s, OH) 3.70 (2H, br s)		4.29 (1H, d, J=11.72) 4.10 (1H, d, J=11.72) 2.14 (2H, d, J=6.35) 2.00 (1H, m) 0.90 (6H, d, J=6.59)

Values are in ppm. The coupling constants (J values) in parentheses are in Hz.

Structure of Seselinol Isovalerate (8) Seselinol isovalerate (**8**) was obtained as a light yellow oil, $[\alpha]_D$ +50.84° ($CHCl_3$), and the molecular formula $C_{19}H_{20}O_5$ was determined by HR-MS. The UV (λ_{max} 270 and 299 nm) spectrum was similar to that of **6**. The ^{1}H-NMR spectrum showed a similar signal pattern to that of seselinol (**6**) (Table IV), except for the following additional signals: δ 4.29 and 4.10 (each 1H, d, J=11.72 Hz), 2.14 (2H, d, J=6.35 Hz), 2.00 (1H, m), 0.90 (6H, d, J=6.59 Hz). Together with these data, the observation of a mass fragment at m/z 213 $[M^+ - CH_2OCOCH_2CH(CH_3)_2]$ and the lower field shift of the methylene protons indicated the presence of an isovaleryl group attached to the hydroxymethyl group of seselinol (**6**). On the basis of these results, seselinol isovalerate can be represented by the formula **8**.

Further investigation of the constituents of this plant is in progress.

Experimental

IR spectra were recorded on a Shimadzu infrared spectrophotometer in $CHCl_3$, UV spectra on a Shimadzu spectrophotometer, optical rotations on a DIP-181 polarimeter (JASCO) in $CHCl_3$, and ^{1}H- and ^{13}C-NMR spectra on a JEOL GX-200 or FX-200, GX-270, GX-400 or JNM-GSX 500 NMR spectrometer (with $SiMe_4$ as an internal standard) in $CDCl_3$, unless otherwise stated. EI- and HR-MS spectra were measured on a Hitachi M-80 or JMS-HX-110 mass spectrometer. NOE enhancements were determined by differential NOE techniques, and ^{1}H–^{13}C COSY and HMBC (J=8 Hz) spectra were recorded on a JEOL GX-400 spectrometer. Column chromatography and centrifugal chromatography were performed on silica gel (Wako gel 60 or Merck Silica gel 60).

Extraction and Isolation The acetone extract (485 g) of dried roots (3.2 kg) of *Citrus hassaku* collected at Innoshima (Hiroshima Prefecture) was subjected to silica gel column chromatography. Elution with hexane, benzene, CH_2Cl_2, acetone and MeOH followed by further separation by preparative thin-layer chromatography (TLC) (solvents: isopropyl ether, hexane–acetone, acetone–$CHCl_3$) gave the following new coumarins: nordenletin (**1**) (53.2 mg), oxanordentatin (**3**) (830.5 mg), oxaclausarin (**4**) (28.4 mg), seselinol (**6**) (3.2 mg) and seselinol isovalerate (**8**) (64.7 mg), along with other compounds.[7]

Nordenletin (1) Light yellow prisms, mp 265—270 °C. $[\alpha]_D$ −61.8° (pyridine, c=0.076). EI-MS m/z: 312, 298, 297, 244, 230, 229 (base peak), 201. FAB-MS m/z: 557 (M^++1). IR ν_{max} cm^{-1}: 3300, 1720, 1620. UV λ_{max} nm: 214, 267, 286, 321 (sh), 343. ^{1}H-NMR (DMSO-d_6, δ): see Table I. ^{13}C-NMR (DMSO-d_6, δ): see Table I.

***O,O'*-Dimethylnordenletin (2)** Nordenletin (**1**) (10 mg) was methylated with diazomethane by a usual method to afford 8 mg of the dimethylated compound (**2**). Colorless oil. EI-MS m/z: 584 (M^+), 569, 513, 368, 292, 256, 222, 221, 164, 149, 129. HR-MS: Calcd for $C_{35}H_{36}O_8$: 584.2410. Found: 584.2415. IR ν_{max} cm^{-1}: 1720, 1600. UV λ_{max} nm: 212, 232 (sh), 254 (sh), 263, 272 (sh), 334. ^{1}H-NMR ($CDCl_3$, δ): 7.95 (1H, d, J=9.76 Hz), 7.67 (1H, d, J=9.77 Hz), 6.47 (1H, d, J=10.01 Hz), 6.35 (1H, dd, J=10.5, 17.58 Hz), 6.28 (1H, d, J=9.76 Hz), 6.09 (1H, d, J=9.77 Hz), 5.54 (1H, d, J=10.01 Hz), 4.98 (1H, dd, J=1.22, 17.58 Hz), 4.93 (1H, m), 4.89 (1H, dd, J=1.22, 10.5 Hz), 3.84, 3.61 (each 3H, s), 2.20 (1H, m), 1.93 (1H, dd, J=8.49, 13.19 Hz), 1.75, 1.70, 1.48, 1.33, 1.32, 0.61 (each 3H, s).

Oxanordentatin (3) Colorless oil, $C_{19}H_{20}O_5$, $[\alpha]_D$ −59.0° (EtOH, c=0.078). EI-MS m/z: 328 (M^+), 313 (base peak), 295, 283, 277, 255, 242, 229. HR-MS: Calcd for $C_{19}H_{20}O_5$: 328.1311. Found: 328.1269. IR ν_{max} cm^{-1}: 3580, 1720, 1605. UV λ_{max} nm: 224, 269 (sh), 293, 338. ^{1}H-NMR, δ: see Table II. ^{13}C-NMR, δ: see Table III.

Oxaclausarin (4) Colorless oil, $C_{24}H_{28}O_5$, $[\alpha]_D$ −32.3° (EtOH, c=0.192). EI-MS m/z: 396 (M^+, base peak), 382, 381, 256, 185, 171, 157, 149, 143, 129. IR ν_{max} cm^{-1}: 3580, 1715, 1603. UV λ_{max} nm: 226, 272 (sh), 284, 304 (sh), 338. ^{1}H-NMR, δ: see Table II. ^{13}C-NMR, δ: see Table III.

Seselinol (6) Light yellow oil, $C_{14}H_{12}O_4$. EI-MS m/z: 244 (M^+), 214, 213 (base peak), 185, 149, 128. HR-MS: Calcd for $C_{14}H_{12}O_4$: 244.0736. Found: 244.0694. $[\alpha]_D$ −111.6° ($CHCl_3$, c=0.108). IR ν_{max} cm^{-1}: 3450, 1730, 1610. UV λ_{max} nm: 218, 283 (sh), 294, 330. ^{1}H-NMR, δ: see Table IV.

Seselinol Isovalerate (8) Light yellow oil, $C_{19}H_{20}O_5$. EI-MS m/z: 328 (M^+), 260, 245, 226, 214, 213 (base peak), 205, 187, 185, 167, 149. $[\alpha]_D$ +50.84° ($CHCl_3$, c=0.118). IR ν_{max} cm^{-1}: 1725, 1600. UV λ_{max} nm: 270, 299. ^{1}H-NMR, δ: see Table IV.

Acknowledgement We are grateful to Mrs K. Suwa and S. Horiyama, Mukogawa Women's University, for measurements of MS and ^{1}H-NMR spectra.

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7) The isolation and characterization of known compounds will be reported elsewhere.

Synthesis of 5,6-, 5,8- and 7,8-Isoquinolinediones from the Corresponding Isoquinolinols and Dimethoxyisoquinolines

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5,8-Isoquinolinediones (12, 18, 25), 7,8-isoquinolinediones (14, 19) and 5,6-isoquinolinediones (26) were synthesized by oxidative demethylation of the corresponding dimethoxyisoquinolines with cerium(IV) ammonium nitrate or silver(II) oxide. 8-Dialkylamino-5,6-isoquinolinediones (31) and 3,5-bis(dialkylamino)-7,8-isoquinolinediones (33) were prepared by copper(II)-catalyzed oxidation of the corresponding isoquinolinols with secondary amines. 5,8-Isoquinolinediones (29, 34, 40) were also prepared.

Keywords synthesis; 5,8-isoquinolinedione; 5,6-isoquinolinedione; 7,8-isoquinolinedione; oxidation; oxidative demethylation; cerium(IV) ammonium nitrate; silver(II) oxide; potassium nitrosodisulfonate

During the past fifteen years, various isoquinolinequinones have been isolated from Actinomycetes and marine sponges. Arai and co-workers have isolated satellite antibiotics, named mimosamycin (**1**)[1] and mimocin (**2**),[2] from *Streptomyces lavendulae*. Mimosamycin (**1**) showed activity against mycobacteria,[1] and was synthesized by using copper(II)-catalyzed autoxidation of 7-hydroxy-6-methylisoquinoline in the presence of morpholine or piperidine.[3] Mimocin (**2**) exhibited strong activity against *Bacillus* (*B.*) *subtilis* and *Candida* (*C.*) *albicans*.[2] In 1979 Faulkner and co-workers described the isolation and the structural determination of renierone (**3**), the major antibacterial metabolite of a marine sponge *Reniera* sp.[4] It showed strong antimicrobial activity against *Staphylococcus* (*S.*) *aureus*, *B. subtilis* and *C. albicans*. Further studies of *Reniera* sp. led to the isolation of **1**, **4**, and **5** from an ethanol extract possessing antimicrobial activity against *S. aureus*, *B. subtilis* and *C. albicans*.[5] In 1987 McKee and Ireland isolated renierol (**6**) from a hard blue sponge, *Xestospongia caycedoi*, which showed antimicrobial activity against *S. aureus* and mild cytotoxicity against L1210 cell line.[6] Furthermore, recently four new isoquinolinequinone metabolites **7**—**10** possessing activity against *B. subtilis* and *S. aureus* have been isolated from a marine sponge, *Xestospongia* sp. and its associated nudibranch *Jorunna funebris*.[7]

We have already described total synthesis of the above biologically active 5,8-isoquinolinediones (**2**—**10**) by using oxidative demethylation of the corresponding 5,8-dimethoxyisoquinolines with cerium(IV) ammonium nitrate (CAN) and silver(II) oxide (AgO), and oxidation of 8-aminoisoquinolines with potassium nitrosodisulfonate (Fremy's salt).[2,8,9] Furthermore, we have reported that 6-methoxy-5,8-quinolinedione and 6-methoxy-7-methyl-5,8-quinolinedione inhibited avian myeloblastosis virus reverse transcriptase to the same extent as streptonigrin, a highly substituted 5,8-quinolinedione, isolated from *Streptomyces flocculus*.[10] The potent biological activities of 5,8-isoquinolinediones (**1**—**10**) and 5,8-quinolinediones have prompted us to undertake the synthesis of various other isoquinolinequinones.

Oxidative demethylation of 5,8-dimethoxyisoquinoline (**11**)[11] with CAN or AgO in aqueous acetonitrile containing pyridine-2,6-dicarboxylic acid *N*-oxide[12] at 0—5 °C for 1—3 h afforded 5,8-isoquinolinedione (**12**) in 72—88% yield. 7,8-Dimethoxyisoquinoline (**13**)[11] was easily oxidized with CAN under the same conditions for 15 min to give unstable 7,8-isoquinolinedione (**14**) in 55% yield. Treatment of 5,7,8-trimethoxyisoquinoline (**17a**) and 5,8-diethoxy-7-methoxyisoquinoline (**17b**) prepared from **15**[13] with CAN at 0—5 °C for 15 min, gave the corresponding *p*-quinone (**18a**) (20—28% yield) and *o*-quinone (**19a**, **b**) (66—70% yield). 5,7,8-Trimethoxy-6-methylisoquinolines (**17c**—**e**)[8] were also oxidatively demethylated with CAN to give the corresponding *p*-quinones (**18c**—**e**) (8—35% yield) and *o*-quinones (**19c**—**e**) (29—44% yield). In contrast, oxidation of **17a**, **b** with AgO at 0—5 °C for 1 h gave the *p*-quinone **18a** as a main product (40—54% yield).

Next, we prepared 6-methoxy-5,8-isoquinolinediones (**25a**—**f**) and 8-methoxy-5,6-isoquinolinediones (**26a**—**f**).

1

2: R = NHCOCOCH$_3$
4: R = H
6: R = OH

3: R = C(CH$_3$)=CH(CH$_3$)
7: R = CH$_3$
8: R = CH$_2$CH$_3$

5: R = C(CH$_3$)=CH(CH$_3$)
9: R = CH$_3$
10: R = CH$_2$CH$_3$

Chart 1

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$Ce(NH_4)_2(NO_3)_6$ (CAN)

11 → 12

CAN

13 → 14

RI, K_2CO_3

15 → 16a, b → 17a, b → (CAN) 18a + 19a, b

16-19
a: $R = CH_3$
b: $R = C_2H_5$

21a → 22a

17c-e → (CAN) 18c-e + 19c-e

17-19
c: R = H
d: R = CN
e: $R = CH_2OBz$

20a-f: X = H
21a-f: X = CHO

20a-f → 23a-f → (R ≠ H) 24a-f → (CAN) 25a-f + 26a-f

20-26
a: R = H
b: $R = CH_3$
c: $R = C_2H_5$
d: $R = C_4H_9$
e: $R = C_6H_{13}$
f: $R = C_8H_{17}$

19a, c, d → 27a, c, d

19, 27
a: $R_1 = R_2 = H$
c: $R_1 = CH_3$, $R_2 = H$
d: $R_1 = CH_3$, $R_2 = CN$

26a, b → 28a, b

26, 28
a: R = H
b: $R = CH_3$

Chart 2

$R-NH_2$

18a → 29

29
a: R = H
b: $R = -(CH_2)_3-$(2-nitrophenyl)

R_1-NH-R_2, $Cu(OAc)_2$, O_2

30 → 31

31
a: $R_1 = R_2 = CH_3$
b: $R_1, R_2 = -(CH_2)_4-$
c: $R_1, R_2 =$ (4-methylpiperidino)

R_1-NH-R_2, $Cu(OAc)_2$, O_2

32 → 33a-d → (CH_3OH, H_2SO_4) 34a-d

33, 34
a: $R_1 = R_2 = CH_3$
b: $R_1, R_2 = -(CH_2)_4-$
c: $R_1, R_2 =$ (4-methylpiperidino)
d: $R_1, R_2 =$ (4-methylpiperazino, N-CH_3)

RI, KOH

32 → 35a, b

MCPBA; $(CH_3)_3SiCN$; $ON(SO_3K)_2$

KNO_3: 35a, b: X = H → 36a, b: $X = NO_2$

37a, b

H_2, Pd/C: 38a, b: $X = NO_2$ → 39a, b: $X = NH_2$

40a, b

35-40
a: $R = C_2H_5$
b: $R = C_4H_9$

Chart 3

TABLE I. Isoquinolinequinones

	Yield[a] (%)	Appearance (Recrystn. solv.)	mp (°C)	Formula	Analysis or HRMS[b] Calcd (Found) C	H	N	MS m/z	IR (KBr) (cm^{-1})	^{1}H-NMR (400 MHz)[c] δ ($CDCl_3$, J=Hz)
5,8-Isoquinolinediones										
12	88 [72]	Yellow needles (CH_2Cl_2–ether)	136—137 (dec.)[d]	$C_9H_5NO_2$	67.93 (68.09	3.17 2.87	8.80 8.59)	159 (M^+, 100) 105 (26)	1665	7.14 and 7.16 (each 1H, d, J=9, C_6-H, C_7-H), 7.91 (1H, d, J=5, C_4-H), 9.12 (1H, d, J=5, C_3-H), 9.32 (1H, s, C_1-H)
18a	20[e] [40]	Yellow needles (CH_2Cl_2–ether)	219—220[f]	$C_{10}H_7NO_3$	63.49 (63.33	3.73 3.59	7.41 7.30)	189 (M^+, 100) 174 (30) 159 (35)	1680 1649	3.95 (3H, s, OCH_3), 6.28 (1H, s, C_6-H), 7.89 (1H, d, J=5, C_4-H), 9.06 (1H, d, J=5, C_3-H), 9.37 (1H, s, C_1-H)
18c	8	Yellow needles (CH_2Cl_2–ether)	130—131	$C_{11}H_9NO_3$	65.02 (65.08	4.46 4.65	6.89 6.72)	203 (M^+, 100)	1670 1658	2.07 (3H, s, C_6-CH_3), 4.18 (3H, s, OCH_3), 7.82 (1H, d, J=5, C_4-H), 8.96 (1H, d, J=5, C_3-H), 9.24 (1H, s, C_1-H)
18d	35	Yellow needles (CH_2Cl_2–ether)	175—177	$C_{12}H_8N_2O_3$	63.16 (62.87	3.53 3.38	12.28 12.12)	228 (M^+, 100) 198 (40)	2240 1680 1648	2.09 (3H, s, C_6-CH_3), 4.26 (3H, s, OCH_3), 8.11 (1H, d, J=5, C_4-H), 9.05 (1H, d, J=5, C_3-H)
18e	18	Yellow needles (CH_2Cl_2–ether)	138—139	$C_{19}H_{15}NO_5$	67.65 (67.58	4.48 4.46	4.15 4.13)	337 (M^+, 4) 105 (100) 77 (32)	1718 1672	2.03 (3H, s, C_6-CH_3), 4.09 (3H, s, OCH_3), 5.91 (2H, s, CH_2), 7.3—7.7 (3H, m) and 8.10 (2H, dd, J=2, 8) for C_6H_5, 7.83 (1H, d, J=5, C_4-H), 8.87 (1H, d, J=5, C_3-H)
25a	29	Yellow needles (CH_2Cl_2–hexane)	181—182	$C_{10}H_7NO_3$	63.49 (63.36	3.73 3.54	7.40 7.31)	189 (M^+, 100) 159 (48)	1690 1648	3.94 (3H, s, OCH_3), 6.23 (1H, s, C_7-H), 7.92 (1H, dd, J=5, 0.7, C_4-H), 9.07 (1H, d, J=5, C_3-H), 9.37 (1H, d, J=0.7, C_1-H)
25b	27 [52]	Yellow needles (Ether–hexane)	110—111	$C_{11}H_9NO_3$	65.02 (65.12	4.46 4.39	6.89 6.74)	203 (M^+, 100)	1673 1653	2.12 (3H, s, C_7-CH_3), 4.16 (3H, s, OCH_3), 7.84 (1H, d, J=5, C_4-H), 9.03 (1H, d, J=5, C_3-H), 9.33 (1H, s, C_1-H)
25c	32 [48]	Yellow needles (Hexane)	57—58	$C_{12}H_{11}NO_3$	66.35 (66.38	5.10 5.02	6.45 6.30)	217 (M^+, 100) 202 (68)	1676 1654	1.13 (3H, t, J=7, CH_2CH_3), 2.64 (2H, q, J=7, CH_2CH_3), 4.16 (3H, s, OCH_3), 7.84 (1H, dd, J=5, 0.6, C_4-H), 9.02 (1H, d, J=5, C_3-H), 9.33 (1H, d, J=0.6, C_1-H)
25d	20 [58]	Yellow needles (Hexane)	37—38	$C_{14}H_{15}NO_3$	68.56 (68.52	6.16 6.18	5.71 5.54)	245 (M^+, 95) 230 (26) 203 (100) 188 (44)	1674 1650	0.94 (3H, t, J=7, CH_2CH_3), 1.35—1.55 (4H, m, $(CH_2)_2CH_3$), 2.61 (2H, t, J=8, CH_2-$(CH_2)_2CH_3$), 4.15 (3H, s, OCH_3), 7.84 (1H, dd, J=5, 0.6, C_4-H), 9.02 (1H, d, J=5, C_3-H), 9.32 (1H, d, J=0.6, C_1-H)
25e	8 [42]	Yellow needles (Hexane)	52—55	$C_{16}H_{19}NO_3$	70.31 (70.25	7.01 7.09	5.12 4.99)	273 (M^+, 100) 204 (48) 203 (89)	1674 1650	0.89 (3H, t, J=7, CH_2CH_3), 1.2—1.55 (8H, m, $(CH_2)_4CH_3$), 2.60 (2H, t, J=8, CH_2-$(CH_2)_4CH_3$), 4.15 (3H, s, OCH_3), 7.85 (1H, dd, J=5, 0.6, C_4-H), 9.02 (1H, d, J=5, C_3-H), 9.33 (1H, d, J=0.6, C_1-H)
25f	11 [46]	Yellow needles (Hexane)	68—70	$C_{18}H_{23}NO_3$	71.73 (71.67	7.69 7.84	4.65 4.53)	301 (M^+, 100) 204 (42) 203 (61)	1674 1650	0.88 (3H, t, J=7, CH_2CH_3), 1.2—1.55 (12H, m, $(CH_2)_6CH_3$), 2.60 (2H, t, J=8, CH_2-$(CH_2)_6CH_3$), 4.14 (3H, s, OCH_3), 7.84 (1H, d, J=5, C_4-H), 9.02 (1H, d, J=5, C_3-H), 9.33 (1H, s, C_1-H)
29a	61	Dark red needles (Methanol)	287—289 (dec.)	$C_9H_6N_2O_2$	62.07 (62.02	3.47 3.22	16.09 16.03)	174 (M^+, 100)	3416 1692	6.02 (1H, s, C_6-H), 7.96 (1H, d, J=5, C_4-H), 8.96 (1H, d, J=5, C_3-H), 9.14 (1H, s, C_1-H)
29b	90	Red plates ($CHCl_3$)	191—192	$C_{18}H_{15}N_3O_4$	64.09 (63.89	4.48 4.40	12.46 12.38)	337 (M^+, 15) 187 (100)	3300 1690 1520 1340	2.11 (2H, quintet, J=7, $CH_2CH_2CH_2$), 3.02 (2H, t, J=7, $ArCH_2$), 3.33 (2H, q, J=7, CH_2NH), 5.83 (1H, s, C_6-H), 6.14 (1H, br, NH), 7.35 and 7.97 (each 1H, dd, J=1.2, 8), 7.40 and 7.56 (each 1H, dt, J=1.2, 8) for C_6H_4, 7.91 (1H, d, J=5, C_4-H), 9.02 (1H, d, J=5, C_3-H), 9.27 (1H, s, C_1-H)
34a	55	Red needles (CH_2Cl_2–hexane)	189—190	$C_{12}H_{12}N_2O_3$ $\cdot$1/10H_2O	61.58 (61.60	5.25 5.04	11.97 11.77)	232 (M^+, 100) 217 (59) 203 (59)	1666 1650	3.29 (6H, s, $N(CH_3)_2$), 3.90 (3H, s, OCH_3), 6.12 (1H, s, C_6-H), 7.04 (1H, s, C_4-H), 8.93 (1H, s, C_1-H)
34b	55	Red needles (CH_2Cl_2–hexane)	202—203	$C_{14}H_{14}N_2O_3$	65.11 (65.00	5.46 5.37	10.85 10.79)	258 (M^+, 100) 230 (38) 229 (97)	1664	2.1 (4H, br s, $CH_2(CH_2)_2CH_2$), 3.55 and 3.75 (each 2H, br s, $CH_2(CH_2)_2CH_2$), 3.90 (3H, s, OCH_3), 6.12 (1H, s, C_6-H), 6.89 (1H, s, C_4-H), 8.93 (1H, s, C_1-H)
34c	48	Orange needles (CH_2Cl_2–hexane)	185—186	$C_{16}H_{18}N_2O_3$	67.12 (66.86	6.34 6.41	9.78 9.81)	286 (M^+, 100) 271 (41) 243 (39) 217 (30)	1666 1646	0.99 (3H, d, J=6, $CHCH_3$), 1.1—1.9 (5H, m, CH_2CHCH_2), 2.9—3.1 (2H, m) and 4.61 (2H, br d, J=13) for $(CH_2)_2N$, 3.89 (3H, s, OCH_3), 6.12 (1H, s, C_6-H), 7.13 (1H, s, C_4-H), 8.90 (1H, s, C_1-H)
34d	66	Orange needles (CH_2Cl_2–hexane)	223—224 (dec.)	$C_{15}H_{17}N_3O_3$ $\cdot$1/5H_2O	61.93 (61.68	6.03 5.93	14.44 14.46)	287 (M^+, 32) 217 (36) 70 (100)	1670 1648	2.39 (3H, s, NCH_3), 2.57 and 3.90 (each 4H, t, J=5, $(CH_2)_2N(CH_2)_2$), 3.90 (3H, s, OCH_3), 6.13 (1H, s, C_6-H), 7.13 (1H, s, C_4-H), 8.91 (1H, s, C_1-H)
40a	54	Yellow needles (CH_2Cl_2–ether)	177—179	$C_{12}H_8N_2O_3$	63.16 (63.07	3.53 3.29	12.28 12.25)	228 (M^+, 100) 184 (51) 172 (85)	2225 1688 1655	1.57 (3H, t, J=7, CH_2CH_3), 4.16 (2H, q, J=7, CH_2CH_3), 6.32 (1H, s, C_6-H), 8.16 (1H, d, J=5, C_4-H), 9.13 (1H, d, J=5, C_3-H)
40b	55	Pale yellow needles (CH_2Cl_2–ether)	148—149	$C_{14}H_{12}N_2O_3$	65.62 (65.47	4.72 4.62	10.93 11.02)	256 (M^+, 41) 200 (33) 172 (100)	2230 1692 1648	1.00 (3H, t, J=7, CH_2CH_3), 1.54 (2H, sextet, J=7, CH_2CH_3), 1.90 (2H, quintet, J=7, $CH_2CH_2CH_3$), 4.08 (2H, t, J=7, CH_2-$(CH_2)_2CH_3$), 6.33 (1H, s, C_6-H), 8.16 (1H, d, J=5, C_4-H), 9.13 (1H, d, J=5, C_3-H)

TABLE I. (continued)

	Yield[a] (%)	Appearance (Recrystn. solv.)	mp (°C)	Formula	Analysis or HRMS[b] Calcd (Found) C	H	N	MS m/z	IR (KBr) (cm^{-1})	^{1}H-NMR (400 MHz)[c] δ ($CDCl_3$, J=Hz)
7,8-Isoquinolinediones										
14	55	Dark red needles (CH_2Cl_2–ether)	170—175 (dec.)	$C_9H_5NO_2$ $\cdot 1/10H_2O$	67.16 (66.88	3.26 3.03	8.70 8.80)	159 (M^+, 26) 131 ($M^+ - CO$, 100) 103 (45)	1698 1680	6.70 and 7.66 (each 1H, d, J=10, C_5-H, C_6-H), 7.52 (1H, d, J=5, C_4-H), 8.93 (1H, d, J=5, C_3-H), 9.18 (1H, s, C_1-H)
19a	66 [17]	Orange needles (CH_2Cl_2–ether)	184—186 (dec.)	$C_{10}H_7NO_3$	63.49 (63.75	3.73 3.61	7.41 7.31)	189 (M^+, 23) 161 ($M^+ - CO$, 100) 103 (59)	1700 1650	4.04 (3H, s, OCH_3), 6.12 (1H, s, C_6-H), 7.72 (1H, d, J=5, C_4-H), 8.93 (1H, d, J=5, C_3-H), 9.24 (1H, s, C_1-H)
19b	70 [25]	Orange prisms ($CHCl_3$–ether)	194—195	$C_{11}H_9NO_3$	65.02 (65.08	4.46 4.30	6.89 6.76)	203 (M^+, 1) 175 ($M^+ - CO$, 98) 147 (88) 106 (100)	1703 1653	1.59 (3H, t, J=7, $CH_2C\underline{H}_3$), 4.26 (2H, q, J=7, $C\underline{H}_2CH_3$), 6.12 (1H, s, C_6-H), 7.78 (1H, d, J=5, C_4-H), 8.97 (1H, d, J=5, C_3-H), 9.27 (1H, s, C_1-H)
19c	44	Orange needles (CH_2Cl_2–ether)	142—144	$C_{11}H_9NO_3$	65.02 (65.10	4.46 4.35	6.89 6.88)	203 (M^+, 1) 175 ($M^+ - CO$, 100) 160 (87)	1700 1665	2.09 (3H, s, C_6-CH_3), 4.02 (3H, s, OCH_3), 7.55 (1H, d, J=6, C_4-H), 7.92 (1H, d, J=6, C_3-H), 9.19 (1H, s, C_1-H)
19d	41	Orange needles (CH_2Cl_2–ether)	183—186 (dec.)	$C_{12}H_8N_2O_3$	63.16 (63.06	3.53 3.34	12.28 12.25)	228 (M^+, 22) 200 ($M^+ - CO$, 80) 185 (100)	1700 1665	2.14 (3H, s, C_6-CH_3), 4.09 (3H, s, OCH_3), 7.80 (1H, d, J=5, C_4-H), 8.93 (1H, d, J=5, C_3-H)
19e	29	Orange needles (CH_2Cl_2–ether)	148—149	$C_{19}H_{15}NO_5$	67.65 (67.70	4.48 4.40	4.15 4.13)	337 (M^+, 1) 309 ($M^+ - CO$, 5) 105 (100) 77 (56)	1727 1688 1650	2.15 (3H, s, C_6-CH_3), 4.08 (3H, s, OCH_3), 5.90 (2H, s, CH_2), 7.3—7.7 (4H, m) and 8.10 (2H, dd, J=2, 8) for C_6H_5 and C_4-H, 8.86 (1H, d, J=4, C_3-H)
33a	51	Red needles (CH_2Cl_2–hexane)	199—201 (dec.)	$C_{13}H_{15}N_3O_2$	63.66 (63.51	6.16 6.12	17.13 16.78)	245 (M^+, 30) 217 ($M^+ - CO$, 100) 202 (58) 188 (78)	1670	3.11 (6H, s, $N(CH_3)_2$), 3.28 (6H, s, $N(CH_3)_2$), 5.99 (1H, s, C_6-H), 6.66 (1H, s, C_4-H), 8.87 (1H, s, C_1-H)
33b	80	Red needles (CH_2Cl_2–hexane)	190—193 (dec.)	$C_{17}H_{19}N_3O_2$	68.67 (68.78	6.44 6.46	14.13 13.98)	299 ($M^+ + 2$, 81) 297 (M^+, 33) 269 ($M^+ - CO$, 89) 240 (100)	1660	2.0—2.2 (8H, m, $2\times CH_2C\underline{H}_2C\underline{H}_2CH_2$), 3.5—3.8 (8H, m, $2\times C\underline{H}_2CH_2CH_2C\underline{H}_2$), 5.89 (1H, s, C_6-H), 6.68 (1H, s, C_4-H), 8.87 (1H, s, C_1-H)
33c	34	Red needles (CH_2Cl_2–hexane)	221—224	$C_{21}H_{27}N_3O_2$ $\cdot 1/5H_2O$	70.64 (70.66	7.73 7.62	11.77 11.58)	355 ($M^+ + 2$, 52) 353 (M^+, 13) 325 ($M^+ - CO$, 100) 98 (58)	1665 1635	1.00 and 1.06 (each 3H, d, J=7, $2\times CHC\underline{H}_3$), 1.1—1.9 (10H, m, $2\times CH_2CHCH_2$), 2.8—3.1 (4H, m), 3.65 (2H, d, J=13) and 4.53 (2H, br d, J=11) for $4\times CH_2N$, 6.06 (1H, s, C_6-H), 6.69 (1H, s, C_4-H), 8.86 (1H, s, C_1-H)
33d	39	Dark red prisms (CH_2Cl_2–ether)	215—218 (dec.)	$C_{19}H_{25}N_5O_2$ $\cdot 1/5H_2O$	63.56 (63.59	7.13 7.11	19.51 19.43)	357 ($M^+ + 2$, 21) 355 (M^+, 23) 327 ($M^+ - CO$, 24) 257 (100)	1664 1636	2.39 (3H, s, NCH_3), 2.41 (3H, s, NCH_3), 2.57 and 2.64 (each 4H, t, J=5, $2\times C\underline{H}_2N(CH_3)$-$C\underline{H}_2$), 3.33 and 3.84 (each 4H, t, J=5, $2\times CH_2NCH_2$), 6.06 (1H, s, C_6-H), 6.69 (1H, s, C_4-H), 8.86 (1H, s, C_1-H)
5,6-Isoquinolinediones										
26a	44	Orange needles (CH_2Cl_2–hexane)	182—183 (dec.)	$C_{10}H_7NO_3$	63.49 (63.21	3.73 3.55	7.40 7.29)	189 (M^+, 5) 161 ($M^+ - CO$, 100) 103 (65)	1712 1642	4.08 (3H, s, OCH_3), 6.07 (1H, s, C_7-H), 7.88 (1H, dd, J=5, 0.7, C_4-H), 8.97 (1H, d, J=5, C_3-H), 9.19 (1H, d, J=0.7, C_1-H)
26b	28 [9]	Orange needles (Ether–hexane)	115—117	$C_{11}H_9NO_3$	65.02 (64.81	4.46 4.42	6.89 6.72)	203 (M^+, 1) 175 ($M^+ - CO$, 100) 160 (54)	1707 1662	2.13 (3H, s, C_7-CH_3), 4.11 (3H, s, OCH_3), 7.81 (1H, d, J=5, C_4-H), 8.88 (1H, d, J=5, C_3-H), 9.04 (1H, s, C_1-H)
26c	14 [12]	Orange needles (Hexane)	95—97	$C_{12}H_{11}NO_3$	66.35 (66.28	5.10 5.01	6.45 6.26)	217 (M^+, 2) 189 ($M^+ - CO$, 59) 174 (100)	1714 1664	1.17 (3H, t, J=8, $CH_2C\underline{H}_3$), 2.61 (2H, q, J=8, $C\underline{H}_2CH_3$), 4.10 (3H, s, OCH_3), 7.82 (1H, dd, J=5, 1, C_4-H), 8.88 (1H, d, J=5, C_3-H), 9.02 (1H, d, J=1, C_1-H)
26d	10 [13]	Red oil		$C_{14}H_{15}NO_3$	245.1052 (245.1067)			245 (M^+, 11) 217 ($M^+ - CO$, 43) 175 (69) 174 (100)	1712 1662	0.95 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.41 (2H, sextet, J=7, $C\underline{H}_2CH_3$), 1.45—1.55 (2H, m, $C\underline{H}_2$-CH_2CH_3), 2.58 (2H, t, J=8, $C\underline{H}_2(CH_2)_2CH_3$), 4.08 (3H, s, OCH_3), 7.81 (1H, dd, J=5, 0.6, C_4-H), 8.87 (1H, d, J=5, C_3-H), 9.00 (1H, d, J=0.6, C_1-H)
26e	7 [17]	Red oil		$C_{16}H_{19}NO_3$	273.1365 (273.1385)			273 (M^+, 10) 245 ($M^+ - CO$, 37) 175 (82) 174 (100)	1712 1664	0.89 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.2—1.6 (8H, m, $(C\underline{H}_2)_4CH_3$), 2.55 (2H, t, J=8, $C\underline{H}_2(CH_2)_4$-CH_3), 4.07 (3H, s, OCH_3), 7.81 (1H, dd, J=5, 0.7, C_4-H), 8.87 (1H, d, J=5, C_3-H), 9.00 (1H, d, J=0.7, C_1-H)
26f	12 [15]	Red oil		$C_{18}H_{23}NO_3$	301.1678 (301.1686)			301 (M^+, 26) 273 ($M^+ - CO$, 54) 175 (95) 174 (100)	1708 1656	0.88 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.2—1.7 (12H, m, $(C\underline{H}_2)_6CH_3$), 2.55 (2H, t, J=8, $C\underline{H}_2$-$(CH_2)_6CH_3$), 4.07 (3H, s, OCH_3), 7.82 (1H, dd, J=5, 0.7, C_4-H), 8.87 (1H, d, J=5, C_3-H), 9.00 (1H, d, J=0.7, C_1-H)
31a	11	Red needles ($CHCl_3$–hexane)	177—178 (dec.)	$C_{11}H_{10}N_2O_2$	65.34 (65.10	4.98 4.83	13.85 13.65)	204 ($M^+ + 2$, 14) 202 (M^+, 7) 174 ($M^+ - CO$, 100)	1690	3.12 (6H, s, $N(CH_3)_2$), 6.00 (1H, s, C_7-H), 7.44 (1H, d, J=6, C_4-H), 8.84 (1H, d, J=6, C_3-H), 9.17 (1H, s, C_1-H)
31b	37	Dark red needles (CH_2Cl_2–hexane)	*ca.* 265 (dec.)	$C_{13}H_{12}N_2O_2$	68.41 (68.29	5.30 5.21	12.27 12.21)	230 ($M^+ + 2$, 24) 228 (M^+, 30) 200 ($M^+ - CO$, 100) 171 (54)	1701	2.1—2.2 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 3.8—3.9 (4H, m, $C\underline{H}_2CH_2CH_2C\underline{H}_2$), 5.93 (1H, s, C_7-H), 7.91 (1H, d, J=5, C_4-H), 8.87 (1H, d, J=5, C_3-H), 9.18 (1H, s, C_1-H)

Table I. (continued)

	Yield[a)] (%)	Appearance (Recrystn. solv.)	mp (°C)	Formula	Analysis or HRMS[b)] Calcd (Found)			MS m/z	IR (KBr) (cm^{-1})	^{1}H-NMR (400 MHz)[c)] δ ($CDCl_3$, J=Hz)
					C	H	N			
31c	60	Red needles (CH_2Cl_2–hexane)	260—262	$C_{15}H_{16}H_2O_2$	70.29 (70.31	6.29 6.27	10.93 10.81)	258 (M^++2, 24) 256 (M^+, 16) 228 (M^+−CO, 100) 199 (31)	1696 1620	1.07 (3H, d, J=6, CHC$\underline{H}_3$), 1.4—2.0 (5H, m, CH_2CHCH_2), 3.09 (2H, dt, J=2, 13) and 3.83 (2H, br d, J=13) for CH_2NCH_2, 6.09 (1H, s, C_7-H), 7.88 (1H, d, J=5, C_4-H), 8.86 (1H, d, J=5, C_3-H), 9.00 (1H, s, C_1-H)

a) Yields by oxidation with AgO in brackets. *b)* HRMS: High-resolution MS. *c)* Measured at 100 MHz (**12**, **14**, **18**, **19**), at 270 MHz (**25a**, **26**, **31**, **33a**, **34**), and in CD_3OD (**29a**). *d)* Lit.[19)] mp 135—138 °C (dec.). *e)* Yields from **17a**. Yields from **17b**: 28% (CAN), 54% (AgO). *f)* Lit.[20)] mp 215—216 °C.

The required 7-alkyl-5,6,8-trimethoxyisoquinolines (**24b—f**) were obtained by employing the modified Pomeranz–Fritsch reaction.[11)] Tosylamides (**23b—f**) prepared from the corresponding aldehydes (**21b—f**) in 87—93% yields, were treated with 6 N hydrochloric acid in dioxane followed by potassium *tert*-butoxide in *tert*-butyl alcohol to give the corresponding isoquinolines (**24b—f**) in 11—31% yields. When concentrated hydrochloric acid, or concentrated hydrochloric acid-zinc chloride was used instead of 6 N hydrochloric acid, the yields of **24c—f** from **23c—f** were remarkably improved (67—89%). However, attempted cyclization of a tosylamide (**23a**) failed. 5,6,8-Trimethoxyisoquinoline (**24a**) was prepared from 2,4,5-trimethoxybenzaldehyde (**21a**) *via* the carbamate **22a** by Hendrickson and Rodriguez's method[14)] in 36% yield. Oxidative demethylation of **24a—f** with CAN containing pyridine-2,6-dicarboxylic acid *N*-oxide at 0—5 °C for 30 min gave the corresponding *p*-quinones (**25a—f**) (8—32% yield) and *o*-quinones (**26a—f**) (7—44% yield). The yields of the quinones (**25a—f** and **26a—f**) decrease as the alkyl group at the C-7 position on the isoquinolines (**24a—f**) is lengthened. In contrast, the isoquinolines (**24b—f**) were smoothly oxidized to the corresponding *p*-quinones (**25b—f**) (42—58% yield) and *o*-quinones (**26b—f**) (9—17% yield) with AgO–nitric acid in dioxane[15)] at 20 °C for 30 min.

The *o*-quinone structures for **19a**, **c**, **d** and **26a**, **b** were further characterized by way of the *o*-phenylenediamine condensation product, *i.e.* pyridophenazines (**27a**, **c**, **d** and **28a**, **b**, respectively).

Treatment of 7-methoxy-5,8-isoquinolinedione (**18a**) with ammonia or 3-(2-nitrophenyl)propylamine[16)] afforded 7-amino-5,8-isoquinolinediones (**29a**, **b**).

Oxidation of 5-isoquinolinol (**30**) with oxygen in the presence of copper acetate and dimethylamine (or pyrrolidine, or 4-methylpiperidine) in methanol gave 8-dialkylamino-5,6-isoquinolinediones (**31a—c**). In contrast 7-isoquinolinol (**32**) was oxidized under the same conditions to 3,5-bis(dialkylamino)-7,8-isoquinolinediones (**33a—d**) in 34—80% yield. The *o*-quinones (**33a—d**) were refluxed with sulfuric acid in methanol to afford the corresponding 3-dialkylamino-7-methoxy-5,8-isoquinolinediones (**34a—d**) in 48—66% yield.

Finally we prepared 1-cyano-5,8-isoquinolinediones (**40a**, **b**). 8-Nitroisoquinolines (**36a**, **b**) prepared by nitration of **35a**,[17)] **b** were treated with *m*-chloroperoxybenzoic acid followed by trimethylsilyl cyanide to afford 1-cyano-8-nitroisoquinolines (**38a**, **b**). Fremy's salt oxidation of 8-amino-1-cyanoisoquinolines (**39a**, **b**) obtained by catalytic reduction of **38a**, **b**, gave 1-cyano-5,8-isoquinolinediones (**40a**, **b**) in 54—55% yield.

The spectral data of 5,8-isoquinolinediones (**12**, **18**, **25**, **29**, **34**, **40**), 7,8-isoquinolinediones (**14**, **19**, **33**) and 5,6-isoquinolinediones (**26**, **31**) are collected in Table I.

We have examined the effects on the growth of lymphoblastoma L5178Y cells, and inhibition of avian myeloblastosis virus reverse transcriptase, human immunodeficiency virus reverse transcriptase and cellular deoxyribonucleic acid (DNA) polymerases α and β of various 5,8-isoquinolinediones (**12**, **18a**, **c**, **d**, **29a**, **b**, and **40a**, **b**), 7,8-isoquinolinediones (**19a**, **c**, **d** and **33a**, **b**) and 5,6-isoquinolinedione (**31a**).[18)] Extensive biological studies are in progress.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured at 100, 270 or 400 MHz, in $CDCl_3$ (unless otherwise noted) with tetramethylsilane as an internal standard. All reactions were run with magnetic stirring. Anhydrous sodium sulfate was used for drying organic solvent extracts, and the solvent was removed with a rotary evaporater and finally under high vacuum. Column chromatography was performed with Silica gel 60 (230—400 mesh).

5,8-Isoquinolinedione (12) (a) A solution of CAN (8.76 g, 16 mmol) in acetonitrile–water (1 : 1, 15 ml) was added dropwise to 5,8-dimethoxyisoquinoline (**11**) (189 mg, 1 mmol) dissolved in acetonitrile–water (1 : 1, 18 ml) containing suspended pyridine-2,6-dicarboxylic acid *N*-oxide (2.93 g, 16 mmol) at 0—5 °C. The mixture was stirred at 0—5 °C for 3 h, adjusted to pH 8 with saturated aqueous $NaHCO_3$ solution, and extracted with CH_2Cl_2 (3 × 30 ml). The extract was washed with water, dried, concentrated to 3 ml, and triturated with ether. The precipitated crystals were collected by filtration. Yield 139 mg (88%).

(b) AgO (620 mg, 5 mmol) was added in portions to **11** (189 mg, 1 mmol) dissolved in acetonitrile–water (1 : 1, 10 ml) containing suspended pyridine-2,6-dicarboxylic acid *N*-oxide (916 mg, 5 mmol) at 0—5 °C. The mixture was stirred at 0—5 °C 1 h, and insoluble compounds were filtered off. The filtrate was diluted with saturated aqueous $NaHCO_3$ solution (20 ml) and extracted with CH_2Cl_2 (3 × 20 ml). The extract was washed with water, dried, and evaporated. The residue was chromatographed using ethyl acetate as the eluent to give 114 mg (72%) of **12**.

7,8-Isoquinolinedione (14) A solution of CAN (2.74 g, 5 mmol) in acetonitrile–water (1 : 1, 10 ml) was added dropwise to 7,8-dimethoxyisoquinoline (**13**) (189 mg, 1 mmol) dissolved in acetonitrile–water (1 : 1, 10 ml) containing suspended pyridine-2,6-dicarboxylic acid *N*-oxide (916 mg, 5 mmol) at 0—5 °C. The mixture was stirred at 0—5 °C for 15 min, adjusted to pH 8 with saturated aqueous $NaHCO_3$ solution, and extracted with CH_2Cl_2 (3 × 30 ml). The extract was washed with water, dried, concentrated to 3 ml, and triturated with ether. The precipitated crystals were collected by filtration. Yield 87 mg (55%).

2,5-Diethoxy-3-methoxybenzaldehyde (16b) Ethyl iodide (5 ml) and K_2CO_3 (2.76 g, 20 mmol) were added to a solution of 2,5-dihydroxy-3-methoxybenzaldehyde (**15**)[13)] (670 mg, 4 mmol) in dry acetone (50 ml). The whole was refluxed for 4 h, and the solid was filtered off. The filtrate was evaporated and the residue was chromatographed (eluting with ethyl

acetate–hexane 3:17) to afford 290 mg (33%) of **16b**, mp 42—43.5 °C (recrystallized from hexane). *Anal.* Calcd for $C_{12}H_{16}O_4$: C, 64.27; H, 7.19. Found: C, 64.20; H, 7.32. ^{1}H-NMR (400 MHz) δ: 1.39 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.41 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.86 (3H, s, OCH_3), 4.04 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.13 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.73 (1H, d, J=3 Hz, C_4-H), 6.84 (1H, d, J=3 Hz, C_6-H), 10.40 (1H, s, CHO).

5,7,8-Trimethoxyisoquinoline (17a) **17a** was prepared from **16a**[21] as described.[11] Yield 46%. mp 85—86 °C (recrystallized from ether). *Anal.* Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.98; N, 6.39. Found: C, 65.67; H, 5.96; N, 6.35. ^{1}H-NMR (270 MHz) δ: 3.98 (3H, s, OCH_3), 4.01 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 6.82 (1H, s, C_6-H), 7.90 (1H, dd, J=6, 1 Hz, C_4-H), 8.42 (1H, d, J=6 Hz, C_3-H), 9.46 (1H, d, J=1 Hz, C_1-H).

5,8-Diethoxy-7-methoxyisoquinoline (17b) **17b** was prepared from **16b** as described.[11] Yield 33%, mp 88—89 °C (recrystallized from ether–hexane). *Anal.* Calcd for $C_{14}H_{17}NO_3$: C, 68.00; H, 6.93; N, 5.66. Found: C, 68.05; H, 6.97; N, 5.64. ^{1}H-NMR (100 MHz) δ: 1.47 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.52 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.95 (3H, s, OCH_3, 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.18 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$, 6.80 (1H, s, C_6-H), 7.92 (1H, d, J=6 Hz, C_4-H), 8.43 (1H, d, J=6 Hz, C_3-H), 9.50 (1H, s, C_1-H).

3-Alkyl-1,2,4-trimethoxybenzene (20c—f) *n*-Butyllithium (16 ml of 1.5 M hexane solution) was added dropwise to a solution of 1,2,4-trimethoxybenzene (**20a**) (3.36 g, 20 mmol) in dry tetrahydrofuran (30 ml) at 0—5 °C. The whole was kept at 0—5 °C for 1 h, then ethyl (or butyl, hexyl, octyl) iodide (22 mmol) in dry tetrahydrofuran (10 ml) was added dropwise at 0—5 °C. The mixture was allowed to warm to room temperature for 30 min, kept for 1.5 h, quenched with water (150 ml), and extracted with ether (3 × 80 ml). The extract was washed with brine, dried, and evaporated. The residue was chromatographed (eluting with ethyl acetate–hexane 1:9) to afford **20c—f** as an oil.

20c: Yield 96%. ^{1}H-NMR (400 MHz) δ: 1.12 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.67 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.78 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 6.55 (1H, d, J=9 Hz, C_5-H), 6.70 (1H, d, J=9 Hz, C_6-H). High-resolution MS (HRMS) Calcd for $C_{11}H_{16}O_3$: 196.1099. Found: 196.1092

20d: Yield 80%. ^{1}H-NMR (400 MHz) δ: 0.92 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.37 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.43—1.53 (2H, m, $C\underline{H}_2CH_2CH_3$), 2.63 (2H, t, J=8 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 3.77 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 6.54 (1H, d, J=9 Hz, C_5-H), 6.69 (1H, d, J=9 Hz, C_6-H). HRMS Calcd for $C_{13}H_{20}O_3$: 224.1412. Found: 224.1436.

20e: Yield 80%. ^{1}H-NMR (400 MHz) δ: 0.88 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.2—1.6 (8H, m, $(C\underline{H}_2)_4CH_3$), 2.62 (2H, t, J=8 Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.77 (3H, s, OCH_3), 3.81 (6H, s, $2\times OCH_3$), 6.54 (1H, d, J=9 Hz, C_5-H), 6.69 (1H, d, J=9 Hz, C_6-H). HRMS Calcd for $C_{15}H_{24}O_3$: 252.1725. Found: 252.1725.

20f: Yield 83%. ^{1}H-NMR (400 MHz) δ: 0.88 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.2—1.6 (12H, m, $(C\underline{H}_2)_6CH_3$), 2.62 (2H, t, J=8 Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.78 (3H, s, OCH_3), 3.81 (6H, s, $2\times OCH_3$), 6.54 (1H, d, J=9 Hz, C_5-H), 6.69 (1H, d, J=9 Hz, C_6-H). HRMS Calcd for $C_{17}H_{28}O_3$: 280.2038. Found: 280.2025.

3-Alkyl-2,4,5-trimethoxybenzaldehyde (21c—f) Phosphoryl chloride (1.5 ml) was added dropwise to dry *N,N*-dimethylformamide (2.5 ml) during 15 min with cooling in ice. The whole was allowed to warm to room temperature, and a solution of **20c—f** (8 mmol) in dry *N,N*-dimethylformamide (2.5 ml) was added. The mixture was heated at 100—110 °C (bath) for 2 h, poured into ice-water, and extracted with CH_2Cl_2 (3 × 20 ml). The extract was washed with water, dried and evaporated. The residue was chromatographed (eluting with ethyl acetate–hexane (1:9—2:8) to afford the aldehyde **21c—f** as an oil.

21c: Yield 75%. ^{1}H-NMR (400 MHz) δ: 1.20 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.69 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.88 (6H, s, $2\times OCH_3$), 3.93 (3H, s, OCH_3), 7.24 (1H, s, C_6-H), 10.28 (1H, s, CHO). HRMS Calcd for $C_{12}H_{16}O_4$: 224.1048. Found: 224.1044.

21d: Yield 74%. ^{1}H-NMR (400 MHz) δ: 0.95 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.41 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.47—1.57 (2H, m, $C\underline{H}_2CH_2CH_3$), 2.64 (2H, t, J=8 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 3.87 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 7.24 (1H, s, C_6-H), 10.28 (1H, s, CHO). HRMS Calcd for $C_{14}H_{20}O_4$: 252.1361. Found: 252.1356.

21e: Yield 70%. ^{1}H-NMR (400 MHz) δ: 0.89 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.2—1.7 (8H, m, $CH_2(C\underline{H}_2)_4CH_3$), 2.63 (2H, t, J=8 Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.86 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 7.24 (1H, s, C_6-H), 10.27 (1H, s, CHO). HRMS Calcd for $C_{16}H_{24}O_4$: 280.1674. Found: 280.1660.

21f: Yield 53%. ^{1}H-NMR (400 MHz) δ: 0.88 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.2—1.7 (12H, m, $CH_2(C\underline{H}_2)_6CH_3$), 2.63 (2H, t, J=8 Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.86 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 7.24 (1H, s, C_6-H), 10.27 (1H, s, CHO). HRMS Calcd for $C_{18}H_{28}O_4$: 308.1987. Found: 308.2018.

***N*-(3-Alkyl-2,4,5-trimethoxybenzyl)-2,2-dimethoxy-*N*-tosylethylamine (23a—f)** **23a—f** was obtained as an oil from 2,4,5-trimethoxybenzaldehyde (**21a**, **b**,[22] **c—f**) according to the reported method.[11]

23a: Yield 92%. ^{1}H-NMR (100 MHz) δ: 2.40 (3H, s, $C_6H_4C\underline{H}_3$), 3.29 (6H, s, $CH(OC\underline{H}_3)_2$), 3.29 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.70 (3H, s, $ArOCH_3$), 3.77 (3H, s, $ArOCH_3$), 3.87 (3H, s, $ArOCH_3$), 4.44 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.44 (2H, s, $ArCH_2N$), 6.40 (1H, s, ArH), 6.82 (1H, s, ArH), 7.22 and 7.61 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$).

23b: Yield 92%. ^{1}H-NMR (100 MHz) δ: 2.17 (3H, s, CH_3), 2.43 (3H, s, $C_6H_4C\underline{H}_3$), 3.25 (6H, s, $CH(OC\underline{H}_3)_2$), 3.28 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.62 (3H, s, $ArOCH_3$), 3.72 (3H, s, $ArOCH_3$), 3.77 (3H, s, $ArOCH_3$), 4.40 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.50 (2H, s, $ArCH_2N$), 6.68 (1H, s, ArH), 7.30 and 7.75 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$). HRMS Calcd for $C_{22}H_{31}NO_7S$: 453.1821. Found: 453.1839.

23c: Yield 92%. ^{1}H-NMR (400 MHz) δ: 1.15 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.42 (3H, s, $C_6H_4C\underline{H}_3$), 2.62 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.23 (6H, s, $CH(OC\underline{H}_3)_2$), 3.27 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.64 (3H, s, $ArOCH_3$), 3.71 (3H, s, $ArOCH_3$), 3.81 (3H, s, $ArOCH_3$), 4.37 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.49 (2H, s, $ArCH_2N$), 6.68 (1H, s, ArH), 7.29 and 7.72 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$). HRMS Calcd for $C_{23}H_{33}NO_7S$: 467.1977. Found: 467.1956.

23d: Yield 92%. ^{1}H-NMR (400 MHz) δ: 0.92 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.38 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.43—1.55 (2H, m, $C\underline{H}_2CH_2CH_3$), 2.42 (3H, s, $C_6H_4C\underline{H}_3$), 2.57 (2H, t, J=8 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 3.23 (6H, s, $CH(OC\underline{H}_3)_2$), 3.26 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.63 (3H, s, $ArOCH_3$), 3.71 (3H, s, $ArOCH_3$), 3.80 (3H, s, $ArOCH_3$), 4.37 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.49 (2H, s, $ArCH_2N$), 6.68 (1H, s, ArH), 7.29 and 7.72 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$). HRMS Calcd for $C_{25}H_{37}NO_7S$: 495.2290. Found: 495.2292.

23e: Yield 93%. ^{1}H-NMR (400 MHz) δ: 0.88 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.25—1.55 (8H, m, $CH_2(C\underline{H}_2)_4CH_3$), 2.42 (3H, s, $C_6H_4C\underline{H}_3$), 2.56 (2H, t, J=8 Hz, $(CH_2)_4CH_3$), 3.23 (6H, s, $CH(OC\underline{H}_3)_2$), 3.26 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.63 (3H, s, $ArOCH_3$), 3.71 (3H, s, $ArOCH_3$), 3.79 (3H, s, $ArOCH_3$), 4.37 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.49 (2H, s, $ArCH_2N$), 6.67 (1H, s, ArH), 7.29 and 7.72 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$). HRMS Calcd for $C_{27}H_{41}NO_7S$: 523.2603. Found: 523.2577.

23f: Yield 87%. ^{1}H-NMR (400 MHz) δ: 0.88 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.2—1.55 (12H, m, $CH_2(C\underline{H}_2)_6CH_3$), 2.42 (3H, s, $C_6H_4C\underline{H}_3$), 2.56 (2H, t, J=8 Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.23 (6H, s, $CH(OC\underline{H}_3)_2$), 3.26 (2H, d, J=6 Hz, $C\underline{H}_2CH$), 3.63 (3H, s, $ArOCH_3$), 3.71 (3H, s, $ArOCH_3$), 3.79 (3H, s, $ArOCH_3$), 4.37 (1H, t, J=6 Hz, $CH_2C\underline{H}$), 4.49 (2H, s, $ArCH_2N$), 6.67 (1H, s, ArH), 7.29 and 7.72 (each 2H, d, J=8 Hz, $C_6\underline{H}_4CH_3$). HRMS Calcd for $C_{29}H_{45}NO_7S$: 551.2916. Found: 551.2927.

5,6,8-Trimethoxyisoquinoline (24a) **24a** was prepared from **21a** as described.[14] Yield 36% (from **21a**). mp 99—100 °C (recrystallized from CH_2Cl_2–hexane). *Anal.* Calcd for $C_{12}H_{13}NO_3$: C, 65.74; H, 5.98; N, 6.39. Found; C, 65.79; H, 6.02; N, 6.32. ^{1}H-NMR (270 MHz) δ: 3.91 (3H, s, OCH_3), 4.04 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 6.68 (1H, s, C_7-H), 7.81 (1H, d, J=6 Hz, C_4-H), 8.46 (1H, d, J=6 Hz, C_3-H), 9.48 (1H, s, C_1-H).

7-Alkyl-5,6,8-trimethoxyisoquinoline (24b—f) A solution of **23b—f** (1 mmol) and 6 N (or concentrated) HCl (2.4 ml) (and $ZnCl_2$ 240 mg) in dioxane (12 ml) was refluxed for 2 h, then cooled, poured into water (50 ml) and extracted with ether (3 × 40 ml). The extract was washed with brine, dried and evaporated. The residue was dissolved in *tert*-butyl alcohol (8 ml), and potassium *tert*-butoxide (673 mg, 6 mmol) was added. The whole was refluxed for 30 min, diluted with water (50 ml), and extracted with CH_2Cl_2 (3 × 30 ml). The extract was washed with water, dried and evaporated. The residue was chromatographed (eluting with ethyl acetate–hexane 2:8—1:1) to afford **24b—f**. The picrates of **24c—f** were prepared by the usual method, and recrystallized from ethanol.

24b: Yield 31% (6 N HCl). mp 52—53 °C (recrystallized from hexane). ^{1}H-NMR (270 MHz) δ: 2.37 (3H, s, C_7-CH_3), 3.94 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 7.85 (1H, dd, J=6, 1 Hz, C_4-H), 8.47 (1H, d, J=6 Hz, C_3-H), 9.38 (1H, d, J=1 Hz, C_1-H). *Anal.* Calcd for $C_{13}H_{15}NO_3$: C, 66.94; H, 6.48; N, 6.00. Found: C, 66.76; H, 6.53; N, 5.86.

24c: Yield 31% (6 N HCl), 84% (concentrated HCl), 89% (concentrated HCl–$ZnCl_2$). Oil. ^{1}H-NMR (400 MHz) δ: 1.25 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.84 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.94 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 4.06 (3H, s, OCH_3), 7.83 (1H, d, J=6 Hz, C_4-H), 8.47 (1H, d, J=6 Hz, C_3-H), 9.37 (1H, s, C_1-H). HRMS Calcd for $C_{14}H_{17}NO_3$: 247.1208. Found: 247.1197. Picrate: mp 186—188 °C. *Anal.* Calcd for $C_{14}H_{17}NO_3\cdot C_6H_3N_3O_7$: C, 50.42; H, 4.23; N, 11.76. Found: C, 50.39;

H, 4.14; N, 11.77.

24d: Yield 11% (6 N HCl), 86% (concentrated HCl), 86% (concentrated HCl–$ZnCl_2$). Oil. ^{1}H-NMR (400 MHz) δ: 0.98 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 1.46 (2H, sextet, $J=7$ Hz, $C\underline{H}_2CH_3$), 1.55—1.65 (2H, m, $C\underline{H}_2CH_2CH_3$), 2.79 (2H, t, $J=8$ Hz, $C\underline{H}_2CH_2CH_2CH_3$), 3.94 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 7.84 (1H, dd, $J=6$, 1 Hz, C_4-H), 8.47 (1H, d, $J=6$ Hz, C_3-H), 9.37 (1H, d, $J=1$ Hz, C_1-H). HRMS Calcd for $C_{16}H_{21}NO_3$: 275.1521. Found: 275.1498. Picrate: mp 158—159°C. *Anal.* Calcd for $C_{16}H_{21}NO_3 \cdot C_6H_3N_3O_7$: C, 52.38; H, 4.80; N, 11.11. Found: C, 52.27; H, 4.73; N, 11.11.

24e: Yield 16% (6 N HCl), 67% (concentrated HCl), 85% (concentrated HCl–$ZnCl_2$). Oil. ^{1}H-NMR (400 MHz) δ: 0.91 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 1.2—1.7 (8H, m, $CH_2(C\underline{H}_2)_4CH_3$), 2.78 (2H, t, $J=8$ Hz, $C\underline{H}_2(CH_2)_4CH_3$), 3.94 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 7.84 (1H, d, $J=6$ Hz, C_4-H), 8.47 (1H, d, $J=6$ Hz, C_3-H), 9.37 (1H, s, C_1-H). HRMS Calcd for $C_{18}H_{25}NO_3$: 303.1834. Found: 303.1828. Picrate: mp 138—139 °C. *Anal.* Calcd for $C_{18}H_{25}NO_3 \cdot C_6H_3N_3O_7$: C, 54.13; H, 5.30; N, 10.52. Found: C, 53.85; H, 5.27; N, 10.54.

24f: Yield 14% (6 N HCl), 71% (concentrated HCl), 81% (concentrated HCl–$ZnCl_2$). Oil. ^{1}H-NMR (400 MHz) δ: 0.89 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 1.2—1.7 (12H, m, $CH_2(C\underline{H}_2)_6CH_3$), 2.78 (2H, t, $J=8$ Hz, $C\underline{H}_2$-$(CH_2)_4CH_3$), 3.94 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 7.83 (1H, dd, $J=6$, 1 Hz, C_4-H), 8.47 (1H, d, $J=6$ Hz, C_3-H), 9.37 (1H, d, $J=1$ Hz, C_1-H). HRMS Calcd for $C_{20}H_{29}NO_3$: 331.2147. Found; 331.2161. Picrate: mp 120—121 °C. *Anal.* Calcd for $C_{20}H_{29}NO_3 \cdot C_6H_3N_3O_7$: C, 55.71; H, 5.75; N, 10.00. Found: C, 55.60; H, 5.81; N, 9.97.

7-Methoxy-5,8-isoquinolinediones (18, 25) and 5-Methoxy- (or 5-Ethoxy)-7,8-isoquinolinediones (19, 26) (a) A solution of CAN (2.74 g, 5 mmol) in acetonitrile–water (1 : 1, 10 ml) was added dropwise to an isoquinoline (**17**, **24**) (1 mmol) dissolved in acetonitrile–water (1 : 1, 10 ml) containing suspended pyridine-2,6-dicarboxylic acid *N*-oxide (0.92 g, 5 mmol) at 0—5 °C. The mixture was stirred at 0—5 °C for 15 min (or 30 min for **24**), adjusted to pH 8 with saturated aqueous $NaHCO_3$ solution, and extracted with CH_2Cl_2 (3 × 30 ml). The extract was washed with water, dried, and evaporated. The residue was chromatographed (eluting with ethyl acetate–CH_2Cl_2 or ethyl acetate–hexane) to afford a less polar *p*-quinone (**18**, **25**) and a more polar *o*-quinone (**19**, **26**).

(b) AgO (620 mg, 5 mmol) was added in portions to **17a**, **b** (1 mmol) dissolved in acetonitrile–water (1 : 1, 10 ml) containing suspended pyridine-2,6-dicarboxylic acid *N*-oxide (916 mg, 5 mmol) at 0—5 °C. The mixture was stirred at 0—5 °C for 1 h, and insoluble compounds were filtered off. The filtrate was diluted with saturated aqueous $NaHCO_3$ solution (20 ml) and extracted with CH_2Cl_2 (3 × 20 ml). The extract was washed with water, dried, and evaporated. The residue was chromatographed. Elution with ethyl acetate–hexane (1 : 1) gave a less polar *p*-quinone (**18a**), and further elution with ethyl acetate–hexane (7 : 3) gave a more polar *o*-quinone (**19a**, **b**).

(c) AgO (495 mg, 4 mmol) and 6 N HNO_3 (1 ml) was added to a solution of **24b**—**f** (1 mmol) in dioxane (15 ml). The whole was stirred for 30 min, diluted with water (50 ml), and extracted with CH_2Cl_2 (3 × 40 ml). The extract was washed with water, dried, and evaporated. The residue was chromatographed. Elution with ethyl acetate–hexane (1 : 4—3 : 7) gave a less polar *p*-quinone (**25b**—**f**), and further elution with ethyl acetate–hexane (2 : 3—1 : 1) gave a more polar *o*-quinone (**26b**—**f**).

Condensation of the *o*-Quinones (19a, c, d and 26a, b) with *o*-Phenylenediamine A mixture of an *o*-quinone (**19a**, **c**, **d** and **26a**, **b**) (0.2 mmol) and *o*-phenylenediamine (22 mg, 0.2 mmol) in ethanol (8 ml) containing acetic acid (0.1 ml) was refluxed for 30 min, and then evaporated. The residue was chromatographed (eluting with ethyl acetate–benzene or ethyl acetate–hexane) to afford the corresponding pyridophenazine (**27a**, **c**, **d** and **28a**, **b**).

27a: Yield 87%. mp 213—214 °C (recrystallized from ethyl acetate–ether). *Anal.* Calcd for $C_{16}H_{11}N_3O$: C, 73.55; H, 4.24; N, 16.08. Found: C, 73.46; H, 3.97; N, 15.80.

27c: Yield 88%. mp 195—196 °C (recrystallized from ethyl acetate–ether). *Anal.* Calcd for $C_{17}H_{13}N_3O$: C, 74.17; H, 4.76; N, 15.26. Found: C, 74.40; H, 4.62; N, 15.32.

27d: Yield 92%. mp 278—279 °C (recrystallized from CH_2Cl_2–ether). *Anal.* Calcd for $C_{18}H_{12}N_4O$: C, 71.99; H, 4.03; N, 18.66. Found: C, 72.23; H, 3.73; N, 18.66.

28a: Yield 97%. mp 246—248 °C (recrystallized from CH_2Cl_2–hexane). *Anal.* Calcd for $C_{16}H_{11}N_3O$: C, 73.55; H, 4.24; N, 16.08. Found: C, 73.24; H, 4.01; N, 16.01.

28b: Yield 94%. mp 204—205 °C (recrystallized from $CHCl_3$–ether). *Anal.* Calcd for $C_{17}H_{13}N_3O$: C, 74.17; H, 4.76; N, 15.26. Found: C, 73.83; H, 4.55; N, 15.09.

7-Amino-5,8-isoquinolinedione (29a) A solution of **18a** (945 mg, 5 mmol) in 10% NH_3–methanol (100 ml) was kept at 40 °C for 1 h, and cooled. The precipitated crystals were collected and recrystallized from methanol to afford 495 mg (57%) of **29a**.

7-(3-(2-Nitrophenyl)propylamino)-5,8-isoquinolinedione (29b) A solution of **18a** (378 mg, 2 mmol) and 3-(2-nitrophenyl)propylamine (432 mg, 2.4 mmol) in methanol (50 ml) was kept at room temperature for 6 h. The precipitated crystals were collected and recrystallized from $CHCl_3$ to afford 608 mg (90%) of **29b**.

8-Dialkylamino-5,6-isoquinolinedione (31) and 3,5-Bis(dialkylamino)-7,8-isoquinolinedione (33) Copper(II) acetate (100 mg) and dimethylamine (0.8 ml of 50% aqueous solution) (or 0.4 ml of pyrrolidine, 4-methylpiperidine, *N*-methylpiperazine) were added to a solution of 5-isoquinolinol **30** (or 7-isoquinolinol (**32**)) (1 mmol) in methanol (10 ml). A steady stream of air was bubbled through this red reaction mixture for 2 h with external ice-cooling. The solvent was removed and the oily residue was dissolved in CH_2Cl_2 (50 ml). The solution was washed with water, dried and evaporated to leave a dark red oil, which was subjected to column chromatography (elution with ethyl acetate–hexane or ethyl acetate-methanol). The crude quinone (**31**, **33**) thus obtained was dissolved in a small amount of CH_2Cl_2, and triturated with hexane to afford the desired product.

3-Dialkylamino-7-methoxy-5,8-isoquinolinedione (34) A solution of **33** (0.2 mmol) in a mixture of methanol (5 ml) and concentrated H_2SO_4 (0.5 ml) was refluxed for 3 h. The reaction mixture was cooled, diluted with ice-water (30 ml), neutralized with pyridine and extracted with CH_2Cl_2 (5 × 20 ml). The extract was washed with water, dried and evaporated. The residue was chromatographed (elution with ethyl acetate–hexane or ethyl acetate–methanol) to afford **34**.

7-Butoxyisoquinoline (35b) A solution of 7-isoquinolinol (**32**) (1.45 g, 10 mmol), KOH (1.35 g, 24 mmol) and butyl iodide (4.41 g, 24 mmol) in butyl alcohol (50 ml) was refluxed for 30 min. The mixture was then cooled, and water (100 ml) was added. The resulting organic layer was separated, dried and evaporated. The residue was chromatographed (eluting with ethyl acetate–hexane 2 : 1) to afford 0.75 g (37%) of **35b** as an oil. ^{1}H-NMR (270 MHz) δ: 1.01 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 1.55 (2H, sextet, $J=7$ Hz, $C\underline{H}_2CH_3$), 1.86 (2H, quintet, $J=7$ Hz, $C\underline{H}_2CH_2CH_3$), 4.11 (2H, t, $J=7$ Hz, $C\underline{H}_2CH_2CH_2CH_3$), 7.21 (1H, d, $J=3$ Hz, C_8-H), 7.36 (1H, dd, $J=3$, 9 Hz, C_6-H), 7.58 (1H, d, $J=6$ Hz, C_4-H), 7.72 (1H, d, $J=9$ Hz, C_5-H), 8.40 (1H, d, $J=6$ Hz, C_3-H), 9.14 (1H, s, C_1-H). HRMS Calcd for $C_{13}H_{15}NO$: 201.1153. Found: 201.1174.

7-Alkoxy-8-nitroisoquinoline (36) A solution of KNO_3 (4.65 g, 0.046 mol) in H_2SO_4 (35 ml) was added dropwise to **35** (0.04 mol) in H_2SO_4 (95 ml) at 0—5 °C. The whole was kept at 0—5 °C for 1.5 h, poured onto ice (400 g), adjusted to pH 8—9 by addition of 20% aqueous NaOH solution and extracted with CH_2Cl_2. The extract was washed with water, dried and evaporated. The residue was chromatographed (eluting with CH_2Cl_2) to afford **36**.

36a: Yield 53%. mp 127—128 °C (recrystallized from CH_2Cl_2–ether). ^{1}H-NMR (100 MHz) δ: 1.48 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 4.32 (2H, q, $J=7$ Hz, $C\underline{H}_2CH_3$), 7.52 (1H, d, $J=9$ Hz, C_6-H), 7.62 (1H, d, $J=6$ Hz, C_4-H), 7.94 (1H, d, $J=9$ Hz, C_5-H), 8.54 (1H, d, $J=6$ Hz, C_3-H), 9.18 (1H, s, C_1-H). *Anal.* Calcd for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.62; N, 12.84. Found: C, 60.44; H, 4.45; N, 12.85.

36b: Yield 58%. mp 74—75 °C (recrystallized from CH_2Cl_2–hexane). ^{1}H-NMR (400 MHz) δ: 0.98 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 1.51 (2H, sextet, $J=7$ Hz, $C\underline{H}_2CH_3$), 1.82 (2H, quintet, $J=7$ Hz, $C\underline{H}_2CH_2CH_3$), 4.25 (2H, t, $J=7$ Hz, $C\underline{H}_2CH_2CH_2CH_3$), 7.55 (1H, d, $J=9$ Hz, C_6-H), 7.64 (1H, d, $J=6$ Hz, C_4-H), 7.95 (1H, d, $J=9$ Hz, C_5-H), 8.55 (1H, d, $J=6$ Hz, C_3-H), 9.18 (1H, s, C_1-H). *Anal.* Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.34; H, 5.66; N, 11.41.

7-Alkoxy-8-nitroisoquinoline *N*-Oxide (37) *m*-Chloroperoxybenzoic acid (80% purity, 4.75 g, 22 mmol) was added to a solution of **36** (20 mmol) in CH_2Cl_2 (100 ml). The whole was stirred at 20 °C for 4 h, and chromatographed. The fraction eluting with ethyl acetate was discarded, and the *N*-oxide **37** was obtained from the fraction eluting with ethyl acetate–ethanol (7 : 3). An analytical specimen was prepared by recrystallization from CH_2Cl_2–ether.

37a: Yield 94%. mp 208—211 °C. ^{1}H-NMR (100 MHz) δ: 1.49 (3H, t, $J=7$ Hz, $CH_2C\underline{H}_3$), 4.36 (2H, q, $J=7$ Hz, $C\underline{H}_2CH_3$), 7.40 (1H, d, $J=9$ Hz, C_6-H), 7.66 (1H, d, $J=7$ Hz, C_4-H), 7.94 (1H, d, $J=9$ Hz, C_5-H), 8.09 (1H, dd, $J=7$, 2 Hz, C_3-H), 8.70 (1H, d, $J=2$ Hz, C_1-H). *Anal.* Calcd for $C_{11}H_{10}N_2O_4$: C, 56.41; H, 4.30; N, 11.96. Found: C, 56.30; H, 4.22; N,

11.72.

37b: Yield 98%. mp 151—152 °C. ^{1}H-NMR (400 MHz) δ: 0.98 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.50 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.82 (2H, quintet, J=7 Hz, $C\underline{H}_2CH_2CH_3$), 4.25 (2H, t, J=7 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 7.40 (1H, d, J=9 Hz, C_6-H), 7.66 (1H, d, J=7 Hz, C_4-H), 7.90 (1H, d, J=9 Hz, C_5-H), 8.05 (1H, dd, J=7, 1.5 Hz, C_3-H), 8.66 (1H, d, J=1.5 Hz, C_1-H). *Anal.* Calcd for $C_{13}H_{14}N_2O_4$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.35; H, 5.22; N, 10.65.

7-Alkoxy-1-cyano-8-nitroisoquinoline (38) Trimethylsilyl cyanide (6 ml, 45 mmol) was added to a suspension of **37** (15 mmol) in 1-methyl-2-pyrrolidinone (30 ml). The whole was kept at 50—60 °C for 20 min, and then 20 °C for 20 h, and diluted with water (150 ml). The precipitated crystals were collected by filtration, washed with water, dried and recrystallized.

38a: Yield 65%. mp 203—204 °C (recrystallized from CH_2Cl_2–ether). ^{1}H-NMR (400 MHz) δ: 1.49 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.39 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.71 (1H, d, J=9 Hz, C_6-H), 7.96 (1H, d, J=6 Hz, C_4-H), 8.11 (1H, d, J=9 Hz, C_5-H), 8.67 (1H, d, J=6 Hz, C_3-H). IR (KBr): 2220 cm^{-1} (CN). *Anal.* Calcd for $C_{12}H_9N_3O_3$: C, 59.26; H, 3.73; N, 17.28. Found: C, 59.21; H, 3.53; N, 17.24.

38b: Yield 64%. mp 94—95 °C (recrystallized from ether–hexane). ^{1}H-NMR (400 MHz) δ: 0.98 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.50 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.84 (2H, quintet, J=7 Hz, $C\underline{H}_2CH_2CH_3$), 4.30 (2H, t, J=7 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 7.70 (1H, d, J=9 Hz, C_6-H), 7.94 (1H, d, J=6 Hz, C_4-H), 8.09 (1H, d, J=9 Hz, C_5-H), 8.66 (1H, d, J=6 Hz, C_3-H). IR (KBr): 2220 cm^{-1} (CN). *Anal.* Calcd for $C_{14}H_{13}N_3O_3$: C, 61.99; H, 4.83; N, 15.49. Found: C, 61.85; H, 4.78; N, 15.45.

7-Alkoxy-8-amino-1-cyanoisoquinoline (39) The 8-nitroisoquinoline (**38**) (2.5 g) in *N*,*N*-dimethylformamide (100 ml) was hydrogenated for 9 h using 10% palladium on carbon (2.5 g) as a catalyst. The catalyst was filtered off and the solvent was removed *in vacuo*. The residue was chromatographed with ethyl acetate–hexane (2 : 3) as the eluent to afford **39**, which was recrystallized from CH_2Cl_2–ether.

39a: Yield 41%. mp 95—96 °C. ^{1}H-NMR (400 MHz) δ: 1.51 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.24 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 5.38 (2H, br, NH_2), 7.27 (1H, d, J=9 Hz, C_6-H), 7.44 (1H, d, J=9 Hz, C_5-H), 7.70 (1H, d, J=6 Hz, C_4-H), 8.37 (1H, d, J=6 Hz, C_3-H). IR (KBr): 2210 cm^{-1} (CN); 3370, 3460 cm^{-1} (NH_2). *Anal.* Calcd for $C_{12}H_{11}N_3O$: C, 67.59; H, 5.20; N, 19.71. Found: C, 67.86; H, 5.08; N, 19.57.

39b: Yield 56%. mp 84—85 °C. ^{1}H-NMR (400 MHz) δ: 1.02 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.56 (2H, sextet, J=7 Hz, $C\underline{H}_2CH_3$), 1.87 (2H, quintet, J=7 Hz, $C\underline{H}_2CH_2CH_3$), 4.17 (2H, t, J=7 Hz, $C\underline{H}_2CH_2CH_2CH_3$), 5.38 (2H, br, NH_2), 7.27 (1H, d, J=9 Hz, C_6-H), 7.45 (1H, d, J=9 Hz, C_5-H), 7.70 (1H, d, J=6 Hz, C_4-H), 8.36 (1H, d, J=6 Hz, C_3-H). IR (KBr): 2215 cm^{-1} (CN); 3370, 3460 cm^{-1} (NH_2). *Anal.* Calcd for $C_{14}H_{15}N_3O$: C, 69.69; H, 6.27; N, 17.41. Found: C, 69.43; H, 6.32; N, 17.34.

7-Alkoxy-1-cyano-5,8-isoquinolinedione (40) A solution of **39** (4 mmol) in acetone (20 ml) was added to a solution of Fremy's salt (5.37 g, 20 mmol) in 1/15 M KH_2PO_4 (225 ml). The mixture was kept at 60 °C for 5 min and then at 20 °C for 1 h, and extracted with CH_2Cl_2 (3 × 120 ml). The extract was washed with 10% HCl (150 ml) and water (150 ml), dried and evaporated. The residue was chromatographed (eluting with CH_2Cl_2) to afford the quinone **40**, which was recrystallized from CH_2Cl_2–ether.

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Tannins and Related Polyphenols of Melastomataceous Plants. II.[1] Nobotanins B, C and E, Hydrolyzable Tannin Dimer and Trimers from *Tibouchina semidecandra* COGN.

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The structures of three new hydrolyzable tannin oligomers, nobotanins B (7), C (20) and E (21), which were isolated from the leaves of *Tibouchina semidecandra* COGN. (Melastomataceae), have been elucidated on the basis of spectral and chemical evidence. Nobotanin B is an isomer of a co-existing dimer, nobotanin F, and nobotanins C and E are trimers possessing the nobotanin B moiety as a partial structure.

Keywords *Tibouchina semidecandra*; Melastomataceae; tannin; ellagitannin oligomer; nobotanin B; nobotanin C; nobotanin E

In the preceding paper, we reported the isolation and structure elucidation of new hydrolyzable tannin dimers, nobotanins A (**5**) and F (**6**) from the leaves of *Tibouchina semidecandra* COGN. (Melastomataceae).[1] Isolation of ten additional hydrolyzable tannin monomers including nobotanin D, 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**1**) and casuarictin (**4**), was also presented.[1] These tannins were the first fully characterized tannins to have been isolated from the Melastomataceous plants. Nobotanins A and F are dimers having unique structures with a valoneoyl group attached to O-4 of one monomer and to O-4/O-6 of the other monomer. Further examination of the leaf extract of this plant resulted in the isolation of an additional dimer, named nobotanin B,[2] and two trimers of related structures, nobotanins C and E.[3] This paper deals with the structure determination of these new oligomeric hydrolyzable tannins.

The new tannins showed the typical coloration with $NaNO_2$–AcOH,[4] and were characterized as ellagitannins composed of the common constituent units [galloyl, valoneoyl, hexahydroxydiphenoyl (HHDP) groups and glucose] based on the acid hydrolysis, which yielded gallic acid (**8**), ellagic acid (**9**), valoneic acid dilactone (**10**) and glucose.

Nobotanin B (**7**), $C_{82}H_{56}O_{52} \cdot 15H_2O$, showed an $(M+Na)^+$ ion peak at *m/z* 1895 in the fast-atom bombardment mass spectrum (FAB-MS). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum (500 MHz) indicated the presence of three galloyl groups [δ 7.27, 7.09, 6.97 (each 2H, s)], a valoneoyl group and two HHDP groups [δ 7.11, 6.62, 6.54, 6.45, 6.44, 6.39, 6.12 (each 1H, s)]. The absolute configurations of the chiroptical HHDP and valoneoyl groups in **7** were determined to be all (*S*), based on the circular dichroism (CD) spectrum, which exhibits a strong positive Cotton effect ($[\theta]$ $+40 \times 10^4$) at 236 nm.[5] In the ^{1}H-NMR spectrum, two anomeric proton signals at δ 6.02 and 6.18 (each d, $J=8.5$ Hz) indicated a dimeric nature of **7**. The other glucose proton signals were assigned with the aid of the ^{1}H–^{1}H shift correlation spectrum (COSY), as summarized in Table I. Their chemical shifts and coupling patterns indicated that both glucose cores in **7** adopt the $^4C_1$ conformation and are fully acylated. The glucose carbon resonances in the ^{13}C-NMR spectrum of **7** are in agreement with those of pterocarinin C (**2**)[6] and casuarictin (**4**),[7] which are the monomeric constituents of nobotanin F (**6**) (Table II). Nobotanin B is, therefore, regarded as an

1 : R=OH, R'=H, R"=G
2 : R=(β)-OG, R'=R"=G
3 : R=(β)-OG, R'=R"=H

G= -CO-(3,4,5-trihydroxyphenyl)

4

5 : R=OH
6 : R=(β)–OG

Chart 1

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isomer of **6**. The characteristic features in the ^{1}H-NMR spectrum of **7** are a remarkable upfield shift of the H-5 signal [δ 3.45 (br d, J=10 Hz)] and a downfield shift of the H-4 signal [δ 5.82 (t, J=10 Hz)], relative to the corresponding signals [δ 4.17 (ddd, J=2, 3.5, 10 Hz) and 5.19 (t, J=10 Hz)] of **6**. These shifts may be reflecting a difference between **6** and **7** in the location of the valoneoyl group linking the two monomer units. Partial hydrolysis of **7** with hot water yielded six hydrolyzates. Among them, four were characterized as isostrictinin (**3**), **12**, **13** and **14** which had also been obtained by similar hydrolysis of nobotanin F (**6**).[1] The ^{1}H-NMR spectrum of the fifth hydrolyzate (**15**) showed the signals due to a galloyl group (δ 6.93 and 6.88, 2H in total) and a dilactonized valoneoyl group [δ 7.54, 7.51 (1H in total), 7.23, 7.22 (1H in total), 7.16, 7.14 (1H in total)]. The duplication of the aromatic and also of the glucose proton signals suggests that **15** exists as an equilibrium mixture of α- and β-anomers, and this is supported by the anomeric proton signals at δ 5.03 (d, J=3.5 Hz) and 4.45 (d, J=8 Hz). The presence of acyl groups at O-4 and O-6 of the glucose core in **15** is evident from the chemical shifts of H-4 [δ 4.99, 4.98 (each t,

7 : R=H
7a: R=Me

8 : R=H
8a : R=Me

9 : R=H
9a : R=Me

10 : R=H
10a : R=Me

11

12 : R=(β)-OG, R'=(*S*)-HHDP
13 : R=OH, R'=(*S*)-HHDP
14 : R=(β)-OG, R'=H, H
15 : R=OH, R'=H, H

16 **17** **18** **19**

Chart 2

Table I. ^{1}H-NMR Data for the Glucose Moieties of **7**, **11** and **21** (500 MHz, Acetone-d_6 + D_2O, J in Hz)

	7	11[a]	20 α-Anomer	20 β-Anomer	21[a]
Glucose-I					
H-1	6.02 d (J=8.5)	5.96 d (J=8.5)	6.10 d (J=8)	6.14 d (J=8)	6.01 d (J=8.5)
H-2	5.18 dd (J=8.5, 10)	5.14 dd (J=8.5, 10)	5.30 dd (J=8, 9.5)	5.17 dd (J=8, 9.5)	5.18 dd (J=8.5, 10)
H-3	5.41 t (J=10)	5.33 t (J=10)	5.32 t (J=9.5)		5.40 t (J=10)
H-4	5.82 t (J=10)	5.80 t (J=10)	5.68 t (J=9.5)		5.83 t (J=10)
H-5	3.45 br d (J=10)	3.49 br d (J=10)	3.37 br d (J=9.5)		3.45 br d (J=10)
H-6	4.92 br d (J=13)	4.84 dd (J=2, 13)	4.87 br d (J=13)		4.91 br d (J=13)
	3.91 br d (J=13)	3.97 d (J=13)	3.79 m		3.91 br d (J=13)
Glucose-II					
H-1′	6.18 d (J=8.5)	6.30 d (J=8.5)	5.98 d (J=8.5)	5.99 d (J=8.5)	6.14 d (J=8.5)
H-2′	5.10 dd (J=8.5, 10)	4.91 dd (J=8.5, 10)	5.16 dd (J=8.5, 9.5)	5.15 dd (J=8.5, 9.5)	5.08 dd (J=8.5, 10)
H-3′	5.79 t (J=10)	5.50 t (J=10)	5.80 t (J=9.5)	5.84 t (J=9.5)	5.80 t (J=10)
H-4′	5.14 t (J=10)	3.90 t (J=10)	5.18 t (J=10)	5.16 t (J=10)	5.10 t (J=10)
H-5′	4.67 dd (J=6.5, 10)	4.01 dd (J=6, 10)	4.58 m		4.60 dd (J=6, 10)
H-6′	5.33 dd (J=6.5, 13)	3.81 dd (J=6, 13)	5.12[b]		5.09 dd (J=6, 13)
	3.92 d (J=13)	3.98 d (J=13)	3.78[b]		3.70 d (J=13)
Glucose-III					
H-1″			5.46 d (J=3.5)	5.07 d (J=8)	6.18 d (J=8.5)
H-2″			5.02 dd (J=3.5, 9.5)	4.80 dd (J=8, 9.5)	5.12 dd (J=8.5, 10)
H-3″			5.58 t (J=9.5)	5.14[b]	5.37 t (J=10)
H-4″			5.50 t (J=9.5)	5.47 t (J=9.5)	5.56 t (J=10)
H-5″			4.38 br d (J=9.5)	3.80[b]	4.09 ddd (J=2, 4, 10)
H-6″			4.14 dd (J=3.5, 12)	4.20 dd (J=3.5, 12)	4.47 br d (J=13)
			4.38 br d (J=12)	4.59 br d (J=12)	4.28 dd (J=4, 13)

a) Measured at 400 MHz in acetone-d_6. *b*) Coupling constants are not clear because of overlapping with other signals.

Table II. ^{13}C-NMR Data for the Glucose Moieties of **7**, **20** and **21**, and Reference Monomers (**2**, **4** and **23**) (126 MHz, Acetone-d_6 + D_2O)

		2	23 α-Anomer	23 β-Anomer	4[a]	7	20[a] α-Anomer	20[a] β-Anomer	21[a]
Glucose-I	C-1	91.9				92.3	92.2		92.4
	C-2	75.3				75.4	75.6	75.4	75.5
	C-3	77.4				78.1	78.1	77.8	78.2
	C-4	67.8				66.9	67.1		67.0
	C-5	73.9				73.9	73.8		74.0
	C-6	62.7				63.4	63.4		63.3
Glucose II	C-1′				92.4	92.3	92.3		92.5
	C-2′				76.0	76.8	76.6	76.5	76.8
	C-3′				77.3	77.0	77.2	77.1	77.0
	C-4′				69.3	69.6	69.6		69.7
	C-5′				73.5	73.4	73.8	73.0	73.5
	C-6′				63.1	63.6	63.4		63.7
Glucose-III	C-1″		91.3	94.9			91.3	94.8	92.5
	C-2″		75.1	77.7			75.1	77.8	75.3
	C-3″		75.4	77.6			75.4	77.3	77.4
	C-4″		68.7	68.5			68.7	68.2	68.0
	C-5″		68.4	73.1			68.3	72.7	74.1
	C-6″		63.1	63.2			63.1		62.7

a) Measured at 100 MHz.

J = 10 Hz)] and H-6 [δ 3.93, 4.02 (each dd, J = 2.5, 13 Hz) and 3.81, 3.86 (each dd, J = 6, 13 Hz)]. Taking the formation of **12**—**14** upon the hydrolysis into consideration, this hydrolyzate was concluded to be represented by the formula **15**. The hydrolyzate (**11**) was characterized as a dimer based on the $(M+Na)^+$ ion peak at m/z 1593 in the FAB-MS, and its ^{1}H-NMR spectrum, which is closely similar to that of **7**, except for the lack of two aromatic 1H singlets due to an HHDP group, and upfield shifts of the H-4′ and H-6′ signals (Table I). This indicates that one of the HHDP groups in **7** was located at O-4′/O-6′ of the glucose core, and consequently the valoneoyl group should be at O-2′/O-3′ of the same glucose core, as depicted in the formula **7**.

On the other hand, methylation of **7** with dimethyl sulfate and potassium carbonate in dry acetone gave an octadecamethyl derivative (**18**), in addition to the permethylated derivative (**7a**), methyl tri-*O*-methylgallate (**8a**), dimethyl hexamethoxydiphenate (**16**) and trimethyl octa-*O*-methylvaloneate (**17**). The ^{1}H-NMR spectrum of **18** indicated the presence of a galloyl group [δ 7.23 (2H, s)], an HHDP group and a valoneoyl group [δ 6.29, 6.67, 6.86, 6.95, 7.15 (each 1H, s)], and also a fully acylated $^{4}C_1$ glucose core (see Experimental). Although these spectral features, together with the electron impact mass spectrum (EI-MS) [m/z 1356 (M^+)], are similar to those of the

permethylated derivative (**19**)[1,8] of rugosin C, **18** is obviously different from **19** in the precise chemical shift of each of the proton signals. Since the result of the above mentioned hydrolysis indicates that O-6/O-4 of one (glucose-I) of the glucose cores in **7** is occupied by the galloyl group/galloyl part of a valoneoyl group, and that the same part in the other glucose core (glucose-II) has an HHDP group, the structure of this degradation product is represented by **18**.

The orientation of the valoneoyl group in nobotanin B was established to be as shown in the formula **7**, based on the ^1H–^{13}C long-range COSY. Among the aromatic proton signals, the HHDP signals (δ 6.62, 6.54, 6.44, 6.39) were readily assigned on the basis of the correlation through the carbonyl carbon signals, correlated with the H-2, H-3, H-4′ and H-6′ signals. The singlet at δ 6.45, assignable to H-3 of the valoneoyl group, was correlated with the signal at δ 169.7 through three-bond coupling, which also showed a cross peak with H-3′ of the glucose core-II. The connectivities, among the galloyl protons and glucose H-1, H-6 and H-1′, were also established through ester carbonyl carbons (Table III).

TABLE III. Connectivities between Aromatic and Glucose Protons through Ester Carbonyl Carbon (*via* Three-Bond Coupling) in the ^1H–^{13}C Long-Range COSY[a] of **7**

Aromatic-H		Carbonyl-C	Glucose-H
Galloyl-H	6.97	164.6	6.02 (H-1)
	7.09	164.8	6.18 (H-1′)
	7.27	167.2	3.91 (H-6)
HHDP-H	6.39	169.7	5.41 (H-3)
	6.44	168.6	5.14 (H-4′)
	6.54	168.0	5.18 (H-2)
	6.62	168.3	3.92 (H-6′)
Valoneoyl H-3	6.45	169.7	5.79 (H-3′)
H-3′	6.12	168.4	[b]
H-6″	7.11	164.8	5.82 (H-4)

a) Measured on a Varian VXR 500; the average J_{CH} value for two- or three-bond coupling was set at 7 Hz. *b*) No cross peak was detected.

Based on these findings, the structure of nobotanin B was concluded to be represented by **7**.

Nobotanin C (**20**), an off-white amorphous powder, $C_{116}H_{80}O_{74}\cdot 22H_2O$, was obtained as the main tannin of this plant. Its trimeric nature was indicated by gel-permeation high-performance liquid chromatography (HPLC),[9] which suggests its molecular weight to be *ca.* 2600. Although the ^1H-NMR spectrum of **20** was complicated owing to the presence of a mixture of α- and β-anomers, the presence of four galloyl, two valoneoyl and two HHDP groups was indicated by four 2H singlets and ten 1H singlets appearing as duplicated signals in the aromatic region (see Experimental). Two pairs of acylated anomeric proton signals were observed at δ 5.98, 5.99 (each d, J=8.5 Hz, 1H in total) and 6.10, 6.14 (each d, J=8 Hz, 1H in total), and the third pair of signals was at δ 5.46 (d, J=3.5 Hz) and 5.07 (d, J=8 Hz). The ^1H–^1H COSY of **20** revealed that the proton signals of two glucose cores (glucose-I and II), whose anomeric centers are acylated, are virtually identical with those of nobotanin B (**7**) (Table I), and the proton signals of the third glucose (glucose-III) are similar to those of praecoxin B (**23**).[1] An analogous correlation was also observed in the ^{13}C-NMR spectrum of **20** which is closely similar to those of **7** and **23**, as shown in Table II.

Partial hydrolysis of **20** with hot water afforded a hydrolyzate (**22**), and **8**, **9**, **11** and **12**—**15**. The ^1H-NMR spectrum of **22**, which showed a duplicated signal for each proton, indicated the presence of two galloyl groups

glucose-III
glucose-II
glucose-I

20 : R=OH
21 : R=(β)-OG

22

20 → a → 22, 12; b → 11, 13; 22 → 13; 12 → 13, 14; 11 → 12; 14 → 15; 13 → 15

Chart 3

[δ 7.26, 7.24 (2H in total), 7.18, 7.16 (2H in total)], an HHDP group and a valoneoyl group [δ 7.05, 6.98 (1H in total), 6.75, 6.70 (1H in total), 6.65, 6.57 (1H in total), 6.42, 6.33 (1H in total), 6.20, 6.12 (1H in total)]. The glucose proton signals showed close similarity to those of nobotanin A (**5**),[1] except for the upfield shifts of the H-1 (δ 5.03, 5.70, each d, J=8 Hz), H-2 (δ 3.63, 3.78, each dd, J=8, 9 Hz) and H-3 (δ 3.88, 4.03, t, J=9 Hz) signals. This hydrolyzate was thus formulated as **22**. The orientation of the valoneoyl group at O-4/O-6 on the glucose core of **22** is regarded as the same as that of nobotanin A (**5**), based on the chemical shifts (δ 6.12, 6.20) of the valoneoyl H-3′, which are similar to those (δ 6.22, 6.32) of **5**. The HPLC analysis of the time course of the hydrolysis revealed that the products formed in an early stage are **22** and **12** (*ca.* 1 : 1), and also **11** and **13** (*ca.* 1 : 1). The amounts of these two sets of products increased in almost the same ratio as the reaction proceeded, indicating the simultaneous cleavages of ester linkages at different positions (a and b in the formula **20**). The rest of the hydrolyzates liberated upon prolonged reaction are thus regarded as products formed from the above four main hydrolyzates as illustrated in Chart 3.

The CD spectrum of nobotanin C (**20**) showed Cotton effects at 227 and 235 nm, of the same signs as and larger amplitudes than those of **5** and **6**,[1] indicating the (S)-configurations for the HHDP and the valoneoyl groups in the molecule.

Based on the above mentioned spectral and chemical evidence, the structure of nobotanin C was determined to be **20**.

Nobotanin E (**21**), $C_{123}H_{84}O_{78} \cdot 20H_2O$, was also shown to be a trimer by a retention volume similar to that of **20** in the gel permeation HPLC. The ^{1}H-NMR spectrum of **21** exhibited signals due to five galloyl [δ 7.29, 7.18, 7.15, 7.11, 6.96 (each 2H, s)], and two HHDP and two valoneoyl groups [δ 7.15, 7.14, 6.50, 6.48, 6.46, 6.41, 6.20, 6.10 (each 1H, s), 6.45 (2H)]. Three anomeric proton signals were observed at δ 6.01, 6.14 and 6.18 (each d, J=8.5 Hz), in accord with the presence of three glucose residues. The signals of each glucose core were unambiguously assigned by ^{1}H–^{1}H COSY as summarized in Table I. The presence of an HHDP group at O-4/O-6 of one (glucose-II) of the glucose cores was shown by the large difference ($\Delta\delta$ 1.39 ppm) between the chemical shifts (δ 5.09 and 3.70) of the C-6 methylene protons.[10] The location of a galloyl group on each O-6 position in the other two glucose cores (glucose-I and III) is shown by the chemical shifts and the coupling patterns of the C-6 methylene protons, which are similar to those of **2**[1] and glucose-I of **7** (Table I). These observations imply that nobotanin E is a trimer possessing nobotanin B (or F) and pterocarinin C moieties. This was further supported by the ^{13}C-NMR spectral comparison with **7** and **2** (Table II).

Upon partial hydrolysis of nobotanin E (**21**) in a boiling-water bath, **12**, **13** and nobotanin C (**20**) were detected by HPLC of the reaction mixture. Nobotanin E was thus indicated to be a gallate of nobotanin C, and this structure was further substantiated by enzymatic degalloylation of **21** with tannase[11] yielding **20**. Consequently, the structure of nobotanin E was determined to be **21**.

It is noticeable that all of the oligomeric hydrolyzable tannins [nobotanins A (**5**), B (**7**), C (**20**), E (**21**) and F (**6**)] isolated from *T. semidecandra* have the casuarictin (**4**), and praecoxin B (**23**) or pterocarinin C (**2**) moieties in each

Chart 4

molecule. They can therefore be regarded as oligomers biogenetically formed by C–O oxidative coupling(s)[12] between the HHDP group(s) of **4** and the galloyl group at O-4 of **23** and/or **2**, as illustrated in Chart 4.

Experimental

General NMR spectra were recorded on Bruker AM 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C), Varian VXR 300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C) and Varian VXR 500 (500 MHz for ^{1}H and 125.6 MHz for ^{13}C) instruments, and the chemical shifts were referenced to acetone-d_6 (2.04 ppm for ^{1}H and 29.8 ppm for ^{13}C). Centrifugal partition chromatography (CPC) was conducted using a CPC Model B-92-N equipped with 12 partition cell cartridges, Type 250W (240 ml in total) (Sanki Engineering). The other instruments ($[\alpha]_D$, ultraviolet (UV), mass spectrum (MS), *etc.*) and chromatographic conditions [HPLC, thin-layer chromatography (TLC), column chromatography] used in this work were the same as those described in the preceding paper.[1]

Isolation of Tannins Nobotanins B (**7**) (277 mg) and E (**21**) (133 mg) were respectively isolated by rechromatographies of fractions G and I from the column chromatography of a part (7.5 g) of the EtOAc extract (13 g) from the aqueous acetone homogenate of leaf extract of *T. semidecandra*.[1] Nobotanin C (**20**) was isolated from a part (6 g) of the *n*-BuOH extract (20 g) obtained from the leaf extract.[1] This *n*-BuOH extract was applied to a column of Diaion HP-20 (3 cm i.d. × 40 cm), which was developed with H_2O containing increasing amounts of MeOH in a stepwise gradient mode. The 50% MeOH eluate (2.61 g) was further chromatographed over Toyopearl HW-40 (coarse grade; 2.2 cm i.d. × 50 cm). Elution with EtOH–H_2O–acetone (6 : 3 : 1) gave crude nobotanin C (675 mg), which was purified by CPC in an ascending mode using the solvent system *n*-BuOH–*n*-PrOH–H_2O (4 : 1 : 5) to afford pure nobotanin C (**20**) (216 mg). Nobotanin C (284 mg) was also isolated by column chromatography [Sephadex LH-20; EtOH–MeOH (4 : 1→1 : 1→3 : 7→2 : 8)] of the MeOH eluate[1] obtained upon column chromatography (Amberlite XAD-2) of the aqueous layer of the leaf extract.

Nobotanin B (**7**): An off-white amorphous powder, $[\alpha]_D$ +54° (c=1.0, MeOH). *Anal.* Calcd for $C_{82}H_{56}O_{52}\cdot 15H_2O$: C, 45.94; H, 4.04. Found: C, 45.46; H, 3.77. FAB-MS: *m/z* 1895 $(M+Na)^+$. UV λ_{max} (MeOH) nm (log ε): 215 (5.23), 275 (4.90). CD (c=0.01, MeOH): $[\theta]_{236}$ $+40\times10^4$, $[\theta]_{262}$ -14×10^4. ^{1}H-NMR: aromatic protons, see text; glucose protons, see Table I. ^{13}C-NMR (126 MHz, acetone-d_6): δ 102.7 [valoneoyl (Val) C-3'], 106.7, 107.1, 107.3, 107.5, 107.7 (HHDP C-3, C-3', Val C-3), 109.6 (Val C-6''), 110.1 (6C, G C-2, C-6), 113.8, 114.2, 114.5, 114.6, 115.7, 116.2, 116.6 (HHDP C-1, C-1', Val C-1, C-1', C-1''), 119.2, 119.4, 120.8 (G C-1), 125.2, 125.5, 125.6, 125.7, 125.8, 126.1 (HHDP C-2, C-2', Val C-2, C-2'), 135.6, 135.7, 136.1, 136.2, 136.5 (2C), 136.6 (HHDP C-5, C-5', Val C-5, C-5', C-2''), 139.1, 139.7, 139.9, 140.1, 140.9 (Val C-3'', C-4'', G C-4), 143.3 (Val C-5''), 144.3, 144.4, 144.5 (2C), 144.8, 144.9, 145.0 (4C), 145.1 (HHDP C-4, C-4', C-6, C-6', Val C-4, C-6, C-6'), 145.8 (2C), 145.9 (2C), 146.1 (2C), (G C-3, C-5), 146.8 (Val C-4'), 164.6, 164.8, 167.2 (G C-7), 168.0, 168.3, 168.6, 169.7 (HHDP C-7, C-7'), 169.7 (Val C-7), 168.4 (Val C-7'), 164.8 (Val C-7''); glucose carbons, see Table II.

Nobotanin C (**20**): An off-white amorphous powder, $[\alpha]_D$ +38° (c=1.0, MeOH). *Anal.* Calcd for $C_{116}H_{80}O_{74}\cdot 22H_2O$: C, 45.61; H, 4.06. Found: C, 45.64; H, 3.81. UV λ_{max} (MeOH) nm (log ε): 223 (5.29), 270 (5.00). CD (c=0.01, MeOH): $[\theta]_{227}$ $+45.7\times10^4$, $[\theta]_{235}$ $+35.6\times10^4$, $[\theta]_{262}$ -12.4×10^4, $[\theta]_{283}$ $+4.5\times10^4$, $[\theta]_{312}$ -4.5×10^4. ^{1}H-NMR (500 MHz, acetone-d_6+D_2O): δ 6.95, 6.97 (each s, 2H in total G), 7.09, 7.10 (each s, 2H in total, G), 7.15 (2H, s, G), 7.24, 7.26 (each s, 2H in total, G), 6.10, 6.14 (each s, 1H in total), 6.17, 6.23 (each s, 1H in total), 6.33, 6.39 (each s, 1H in total), 6.43, 6.45 (each s, 1H in total), 6.46 (1H, s), 6.47, 6.50 (each s, 1H in total), 6.63, 6.68 (each s, 1H in total), 6.96, 6.93 (each s, 1H in total), 7.07, 7.10 (each s, 1H in total), 7.14, 7.11 (each s, 1H in total) (HHDP and Val); glucose protons, see Table I. ^{13}C-NMR, see Table II.

Nobotanin E (**21**): An off-white amorphous powder, $[\alpha]_D$ +49° (c=1.0, MeOH). *Anal.* Calcd for $C_{123}H_{84}O_{78}\cdot 20H_2O$: C, 46.60; H, 3.94. Found: C, 46.51; H, 3.63. UV λ_{max} (MeOH) nm (log ε): 219 (5.43), 273 (5.09). ^{1}H-NMR: aromatic protons, see text and glucose protons, see Table I. ^{13}C-NMR (100 MHz, acetone-d_6+D_2O): δ 103.0, 105.0 (Val C-3'), 1070. 107.5 (3C), 107.8 (2C) (HHDP C-3, C-3', Val C-3), 110.1, 111.1 (Val C-6''), 114.3 (2C), 114.4, 114.7 (2C), 114.9, 115.5, 115.8, 116.7, 117.3 (HHDP C-1, C-1', Val C-1, C-1', C-1''), 120.0, 120.1, 120.3, 121.4, 121.6 (G C-1), 125.4, 126.1 (2C), 125.2, 126.3 (2C), 126.4, 126.5 (HHDP C-2, C-2', Val C-2, C-2'), 135.5, 135.8, 136.1 (2C), 136.2, 136.4 (2C), 136.5 (2C), 136.7 (HHDP C-5, C-5', Val C-5, C-5', C-2''), 138.9, 139.0, 139.5, 139.8 (2C), 140.1, 140.2, 140.6 (2C) (G C-4, Val C-3'', C-4''), 143.3, 143.6 (Val C-5''), 144.4 (2C), 144.6 (2C), 144.8, 144.9, 145.0, 145.1 (3C), 145.2 (3C), 145.3 (HHDP C-4, C-4', C-6, C-6', Val C-4, C-6, C-6'), 145.8 (4C), 145.9 (2C), 146.1 (4C) (G C-3, C-5), 146.9, 147.0 (Val C-4'), 164.3, 164.5, 164.7, 165.0, 165.1, 166.5, 167.4, 167.6, 167.9, 168.3, 168.5, 168.6, 169.6 (2C), 169.7 (ester carbonyl); glucose carbons, see Table II.

Acid Hydrolysis of Nobotanins B (7), C (20) and E (21) A solution of **7** (3 mg) in 5% sulfuric acid (0.8 ml) was heated in a boiling-water bath for 5 h. After cooling, the reaction mixture was extracted with AcOEt. The aqueous layer was neutralized with ion exchange resin (Amberlite IRA-410), filtered and evaporated to dryness. The sugar component was identified as glucose by gas liquid chromatography (GLC) [2.5% OV-1, column temperature 170 °C] after trimethylsilylation. The AcOEt-soluble portion was evaporated, and methylated with an excess of ethereal CH_2N_2. The residue was subjected to preparative TLC (SiO_2, ligroin–$CHCl_3$–acetone 6 : 3 : 1) to yield **8a**, **9a** and **10a** which were identified by co-chromatography (TLC) with authentic samples and MS.

Nobotanins C (**20**) and E (**21**) were similarly hydrolyzed to give the same products as described above.

Partial Hydrolysis of Nobotanin B (7) An aqueous solution (100 ml) of **7** (95 mg) was refluxed under a nitrogen atmosphere for 7 h. The concentrated solution was submitted to column chromatography over Toyopearl HW-40 (fine grade, 2.2 cm i.d. × 70 cm) developing with 70% aqueous MeOH and MeOH. The eluate from 70% aqueous MeOH gave isostrictinin (**3**) (12 mg), **15** (2 mg), **12** (3 mg), **14** (3 mg) and **13** (2 mg). The MeOH eluate yielded the hydrolyzate (**11**) (8 mg).

Hydrolyzate (**15**): A light brown amorphous powder, $[\alpha]_D$ +33° (c=0.1, MeOH). UV λ_{max} (MeOH) nm (log ε): 217 (4.58), 257 (4.42), 360 (3.66). ^{1}H-NMR (400 MHz, acetone-d_6): δ 6.93, 6.88 (each s, 2H in total, G), 7.54, 7.51 (1H in total), 7.23, 7.22 (1H in total), 7.16, 7.14 (1H in total) (dilactonized valoneoyl), 5.03 [d, J=3.5 Hz, H-1 (α)], 4.45 [d, J=8 Hz, H-1 (β)], 3.36 [d, J=3.5, 10 Hz, H-2 (α)], 3.18 [dd, J=8, 10 Hz, H-2 (β)], 3.48, 3.70 [each t, J=10 Hz, H-3 (α and β)], 4.98, 4.99 [each t, J=10 Hz, H-4 (α and β)], 3.56, 3.93 [each ddd, J=2.5, 6, 10 Hz, H-5 (α and β)], 3.93, 4.02 [each dd, J=2.5, 13 Hz, H-6 (α and β)], 3.81, 3.86 [each dd, J=6, 13 Hz, H-6' (α and β)].

Hydrolyzate (**11**): An off-white amorphous powder, $[\alpha]_D$ +31° (c=0.4, MeOH). FAB-MS: *m/z* 1593 $(M+Na)^+$. *Anal.* Calcd for $C_{68}H_{50}O_{44}\cdot 7H_2O$: C, 48.08; H, 3.77. Found: C, 47.96; H, 3.74. UV λ_{max} (MeOH) nm (log ε): 222 (4.99), 275 (3.65). ^{1}H-NMR (400 MHz, acetone-d_6): δ 7.27, 7.09, 6.95 (each 2H, s, G), 7.12, 6.72, 6.47, 6.45, 6.08 (each 1H, s, HHDP and Val); glucose protons, see Table I.

Methylation of Nobotanin B (7) A mixture of **7** (100 mg), potassium carbonate (700 mg) and dimethyl sulfate (1 ml) in dry acetone (30 ml) was stirred at room temperature and refluxed for 48 h. After filtration to remove inorganic salt, the solvent was evaporated off, and the syrupy residue was purified by preparative TLC (SiO_2, ligroin–benzene–acetone 1 : 4 : 1) to yield methyl tri-*O*-methylgallate (**8a**) (2 mg), dimethyl hexamethoxydiphenate (**16**) (1 mg), trimethyl octa-*O*-methylvalonate (**17**) (1 mg), the octadecamethyl derivative (**18**) (2 mg) and permethylated nobotanin B (**7a**) (33 mg).

18: A white amorphous solid, EI-MS: *m/z* 1356 (M^+). ^{1}H-NMR (400 MHz, acetone-d_6): δ 6.18 (d, J=8.5 Hz, H-1), 5.18 (dd, J=8.5, 9.2 Hz, H-2), 5.51 (dd, J=9.2, 10 Hz, H-3), 5.12 (t, J=10 Hz, H-4), 4.57 (dd, J=6, 10 Hz, H-5), 5.26 (dd, J=6, 13 Hz, H-6), H-6' is overlapped by OMe signals, 3.65, 3.68, 3.70, 3.71, 3.72, 3.73, 3.74, 3.82, 3.83, 3.85, 3.86, 3.87, 3.89, 3.90, 3.91, 4.00 (each 3H, s), 3.84 (6H, s) (18 × OMe), aromatic protons, see text.

Permethylated Derivative (**7a**): A white amorphous solid, ^{1}H-NMR (400 MHz, acetone-d_6): δ 7.35, 6.95, 6.89 (each 2H, s, G), 6.96, 6.86, 6.79, 6.71, 6.69, 6.67, 6.61 (each 1H, s, HHDP and Val), 6.12 (d, J=8.5 Hz, H-1), 5.43 (dd, J=8.5, 10 Hz, H-2), 5.19 (t, J=10 Hz, H-3), 5.74 (t, J=10 Hz, H-4), 4.32 (dd, J=2, 10 Hz, H-5), 4.26 (dd, J=2, 13 Hz, H-6), 4.27 (d, J=13 Hz, H-6), 6.38 (d, J=8.5 Hz, H-1'), 5.33 (dd, J=8.5, 9 Hz, H-2'), 6.07 (dd, J=9, 10 Hz, H-3'), 5.11 (t, J=10 Hz, H-4'), 4.86 (dd, J=6, 10 Hz, H-5'), 5.54 (dd, J=6, 13 Hz, H-6'), 4.29 (d, J=13 Hz, H-6'), 3.93, 3.91, 3.90, 3.87, 3.86, 3.82, 3.74, 3.70, 3.68, 3.65, 3.64, 3.63 (each 3H, s), 3.89, 3.88, 3.85, 3.77, 3.73, 3.69, 3.67 (each 6H, s), 3.72 (9H, s) (29 × OMe).

Partial Hydrolysis of Nobotanin C (20) A solution of nobotanin C (**20**) (300 mg) in water (300 ml) was refluxed under nitrogen atmosphere for 18 h. The reaction mixture was then concentrated and the residue was chromatographed over Sephadex LH-20. Elution with EtOH gave gallic acid (**8**) (14 mg), ellagic acid (**9**) (16 mg), **15** (4 mg), **14** (6 mg) and **13** (9 mg). Elution with EtOH–MeOH (9 : 1) yielded **12** (25 mg). The

EtOH–MeOH (7 : 3) eluate gave **11** (5 mg) and **22** (10 mg).

Hydrolyzate (**22**): A light brown amorphous powder, $[\alpha]_D$ +40° (c=0.2, MeOH). UV λ_{max} (MeOH) nm (log ε): 223 (4.84), 270 (4.51). ^{1}H-NMR (400 MHz, acetone-d_6) (α- and β-anomer *ca.* 1 : 1): δ 7.26, 7.24 (2H in total, G), 7.18, 7.16 (2H in total, G), 7.05, 6.98 (1H in total), 6.75, 6.70 (1H in total), 6.65, 6.57 (1H in total), 6.42, 6.33 (1H in total), 6.20, 6.12 (1H in total) (HHDP and Val), 5.03, 5.70 [each d, J=8 Hz, H-1 (α and β)], 3.63, 3.78 [each dd, J=8, 9 Hz, H-2 (α and β)], 3.88, 4.03 [each t, J=9 Hz, H-3 (α and β)], 4.92, 4.97 [each t, J=9 Hz, H-4 (α and β)], 4.06, 4.12 [each dd, J=6.5, 9 Hz, H-5 (α and β)], 5.12, 5.09 [each dd, J=6.5, 14 Hz, H-6 (α and β)], 3.72, 3.64 [each d, J=14 Hz, H-6 (α and β)], 5.46 (d, J=3.5 Hz, H-1′ (α)], 5.31 [d, J=8 Hz, H-1′ (β)], 5.02 [dd, J=3.5, 10 Hz, H-2′ (α)], 4.84 [dd, J=8, 10 Hz, H-2′ (β)], 5.69, 5.62 [each t, J=10 Hz, H-3′ (α and β)], 5.21, 5.58 [each t, J=10 Hz, H-4′ (α and β)], 4.21, 4.14 [each br d, J=10 Hz, H-5′ (α and β)], 5.54, 4.72 [each d, J=13 Hz, H-6′ (α and β)], 3.96, 4.13 [each dd, J=2, 13 Hz, H-6′ (α and β).

Partial Hydrolysis of Nobotanin E (21) An aqueous solution (2 ml) of **21** (1 mg) was refluxed on a hot plate for 2 h, and the reaction mixture was analyzed by normal phase HPLC. Nobotanin C (**20**) and **12** accompanied with a small amount of **13** were detected.

Enzymatic Hydrolysis of Nobotanin E (21) An aqueous solution (5 ml) of **21** (20 mg) was incubated with tannase (10 drops) at 37 °C for 17 h. After removal of the solvent, the residue was chromatographed over Sephadex LH-20 (1.1 cm i.d. × 27 cm) to give **8** (7 mg) and nobotanin C (**20**) (4 mg), which were identified by ^{1}H-NMR spectral comparisons.

Acknowledgements The authors are grateful to Dr. N. Toh, Faculty of Engineering, Kyushu Kyoritsu University, for CD measurements, and to Mr. S. Iwadow, Faculty of Pharmaceutical Sciences, Okayama University for FAB-MS measurements. The VXR 500 NMR instrument is the property of the SC-NMR Laboratory of Okayama University.

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Stereochemistry of Novel Triterpenes from *Cynanchum hancokianum*

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The absolute stereochemistry of hancokinol, hancolupenone, hancolupenol, hancolupenol hexacosanoate, which are novel triterpenes, from *Cynanchum hancokianum*, has been elucidated by means of spectroscopic [nuclear magnetic resonance (NMR), circular dichroism (CD)] analyses.

Keywords absolute stereochemistry; hancokinol; hancolupenone; hancolupenol; hancolupenol hexacosanoate; triterpene; *Cynanchum hancokianum*; spectroscopic (NMR, CD) analyses

Cynanchum hancokianum (MAXIM.) AL. ILJINSKI. (Asclepiadaceae), distributed in Inner Mongolia, is known as a Chinese folk medicine possessing antitumor activity. We previously obtained two phenanthroindolizine alkaloids, antofine and de-6-*O*-methylantofine, from the hydrochloric acid extract of this plant.[1] Our further investigations have led to the isolation of four new pentacyclic triterpenes, named hancokinol (**1**),[2] hancolupenone (**2**),[3] hancolupenol (**3**)[3] and hancolupenol hexacosanoate (**4**), from the ethanol extract of the same plant source. Compounds **1**—**4** were obtained on chromatography (silica gel) of the petroleum ether-soluble portion of the ethanol extract, by elution with a mixture of petroleum ether and acetone. This paper deals in detail with the structural elucidation of these compounds by means of spectroscopic (proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR), circular dichroism (CD)) analyses.

Hancokinol (**1**),[2] $C_{30}H_{50}O$, mp 229—230 °C (MeOH), $[\alpha]_D^{20}$ +16.2° ($CHCl_3$), showed an infrared (IR) hydroxyl band at 3580 cm^{-1}.

Distortionless enhancement by polarization transfer (DEPT) experiments indicated the presence of eight methyls, nine methylenes, seven methines and six quaternary carbons. Protons and carbons were assigned by two-dimensional (2D) NMR spectroscopy, and the parameters are listed in Table I. The position of two *gem*-methyls (C-23, C-24) and four angular methyls (C-25—C-28) were unambiguously determined by the 2D correlations to the surrounding protonated or quaternary carbons. The presence of an isopropyl comprised of two *sec*-methyls at δ_H 0.87 (H_3-29) and 0.90 (H_3-30) and a methine at δ_H 1.49 (H-20) was confirmed by ^{1}H–^{1}H correlation spectroscopy (COSY) experiments. A methine at δ_H 1.57 (H-19) was correlated to a methine at δ_H 1.48 (H-18) and H-20, leading to iso-Pr-19 (^{1}H–^{1}H). A one-proton signal at δ_H 5.54 was attributed to a trisubstituted olefinic proton (H-6) possessing a neighboring methylene at δ_H 2.26 and 2.01 (H_2-7) (^{1}H–^{1}H). ^{1}H–^{1}H and ^{1}H–^{13}C COSY experiments placed a hydroxymethine (δ_H 3.47, δ_C 76.58, C-3) between a methylene (δ_H 1.90, 1.69, δ_C 28.81, C-2) and a quaternary carbon (δ_C 41.32, C-4) possessing two *gem*-methyls (δ_H 1.04, δ_C 27.26, C-23) and (δ_H 1.13, δ_C 25.39, C-24). The hydroxyl was assigned to be axial on the basis of the coupling (t, J=2.5 Hz) observed for H-3. The correlations of two methines at δ_H 1.48 (H-8) and 2.35 (H-10) to neighboring methylenes at δ_H 2.26 (H-7), and 1.59 and 1.42 (H_2-1) were respectively observed in the ^{1}H–^{1}H COSY spectrum.

The electron impact high resolution mass spectrum

Chart 1

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TABLE I. NMR Data for **1**

Carbon No.	δ_C	Correlated H[a] δ_H	H coupled with C[b]	H coupled with H[c]
1	20.80 t	Hα-1 1.42 m	H_2-2, H-3, H-10	Hβ-1, Hβ-2, H-10
		Hβ-1 1.59 m		Hα-1, H_2-2, H-10
2	28.81 t	Hα-2 1.90 ddt (2.5, 4.0, 14.0)		Hβ-1, Hβ-2, H-3
		Hβ-2 1.69 m		H_2-1, Hα-2, H-3
3	76.58 d	H-3 3.47 t (2.5)	Hβ-1, H_3-23	H_2-2
4	41.32 s		Hβ-2, H_3-23, H_3-24	
5	140.64 s		H_3-23, H_3-24	
6	121.47 d	H-6 5.54 dt (6.0, 2.0)	H_2-7, H-8	H_2-7
7	22.65 t	Hα-7 2.01 dd (19.0, 6.0)	H-8	H-6, Hβ-7
		Hβ-7 2.26 ddd (19.0, 3.2, 2.0)		H-6, Hα-7, H-8
8	44.54 d	H-8 1.48 m	Hβ-7, H_3-25, H_3-26	Hβ-7
9	34.49 s		H-8, H_2-11, H_3-25	
10	37.23 d	H-10 2.35 d (12.0)	H-8, H_3-25	H_2-1
11	30.64 t	H_2-11 1.13 m	H-8, H_3-25	H_2-12
12	30.72 t	Hα-12 1.59 m		H_2-11, Hβ-12
		Hβ-12 1.36 m		H_2-11, Hα-12
13	39.25 s		H-8, H_2-11, Hα-12, H-18, H_3-26, H_3-27	
14	39.62 s		Hβ-7, H-8, H-18, H_3-26, H_3-27	
15	29.38 t	H_2-15 1.25 m	H-8, H_2-16, H_3-26	H_2-16
16	32.65 t	H_2-16 1.49 m	Hβ-22, H_3-28	H_2-15
17	39.92 s		H_2-15, H_2-16, Hb-21, Hβ-22, H_3-28	
18	54.46 d	H-18 1.48 d (2.0)	Hα-12, Hb-21, Hα-22, H_3-27, H_3-28	H-19
19	48.24 d	H-19 1.57 m	Hb-21, Hα-22, H_3-29, H_3-30	H-18, H-20, Ha-21
20	35.96 d	H-20 1.49 m	H-18, H_3-29, H_3-30	H_3-29, H_3-30
21	27.85 t	Ha-21 1.44 m	H-18, H-20, Hβ-22	H-19, Hb-21, Hα-22
		Hb-21 1.67 m		Ha-21, Hβ-22
22	42.44 t	Hα-22 1.25 m	H-18, H_3-28	Ha-21, Hβ-22
		Hβ-22 1.79 dd (11.5, 9.0)		Hb-21, Hα-22
23	27.26 q	H_3-23 1.04 s	H_3-24	
24	25.39 q	H_3-24 1.13 s	H_3-23	
25	28.77 q	H_3-25 0.90 s	Hβ-1, H-8, H-10, H_2-11, H_2-12	
26	15.39 q	H_3-26 0.80 s	H-8, H_2-15	H-8
27	16.78 q	H_3-27 0.88 s	H_2-11, Hβ-12, H-18	
28	33.35 q	H_3-28 0.93 s	H_2-16, H-18, H_2-22	
29[d]	21.32 q	H_3-29 0.87 d (6.8)	H_3-30	H-20
30[d]	23.44 q	H_3-30 0.90 d (6.0)	H_3-29	H-20

a) ^1H–^{13}C (one-bond) COSY. Figures in parentheses are coupling constants (Hz). *b)* ^1H–^{13}C (long-range) COSY. *c)* ^1H–^1H COSY. *d)* These are exchangeable.

(EIHRMS) displayed the characteristic fragments at *m/z* 152.1205 (152.1201 for $C_{10}H_{16}O$) (a) and 274.2648 (274.2660 for $C_{20}H_{34}$) (b) arising from a retro-Diels–Alder fission, regarded as suggestive of a Δ^5-compound.[4] Thus, these findings and the NMR data pointed out a 9,13-dimethyl-25,26-dinorlup-5-en-3-ol for **1**.

The nuclear Overhauser effect (NOE) data are given in Table II. The relative stereochemistry was examined assuming a chair form for each ring-A, -C and -D and a half-chair form for ring-B and using a basis of Hα-10. NOEs observed among H-2 (δ_H 1.90), H-10 and H_3-23 suggested their orientations to be axial (α) with respect to ring-A. The mutual enhancements among H-10, H_3-23 and H_3-26 pointed out C(8)-C(14) to be axial (α) and consequently to be H-8 equatorial (β) with respect to ring-B. An NOE observed between H-8 and H_3-25 led to Meβ (ax)-9 (H_3-25) with respect to ring-B, resulting in B/C *cis*. The enhancements linking H-18 to H_3-26 and H_3-28 suggested Meα (ax)-14 (H_3-26), Meα (eq)-17 (H_3-28) and Hα (ax)-18 with respect to ring-D. An NOE observed between Hβ-22 (δ_H 1.79) and H_3-27 displayed Me-13 (H_3-27) and C(17)-C(22) to be axial (β) with respect to ring-D. Thus, C/D *trans* and D/E *cis* were established on the basis of Meβ (ax)-13, Meα (ax)-14, Meα (eq)-17 and Hα (ax)-18. Unfortunately, the orientation of iso-Pr-19 could not be decided by NOE experiments.

A Dreiding model based on the NOE data indicated the presence of a steric repulsion between H_3-23 and H_3-26, so that ring B would deviate from a half-chair form. Actually, an NOE observed between Hα-7 (δ_H 2.01) and H-10 is only explainable by an envelope form with C(9) out of the plane or a half-boat form (probably impossible in practice) for ring-B. The ring-B deformation would be transmitted to ring-A, causing mutual approaches between H-3 and H-10 and between Hβ-1 (δ_H 1.59) and H_3-24. Such steric situations would be responsible for the observed NOEs between them.

TABLE II. NOE Data for **1**

Irradiated H	Observed H (%)
Hβ-1	H_3-24 (1.5), H_3-25 (1.5)
H-3	Hα-2 (5.1), Hβ-2 (4.6), H_3-23 (1.9), H_3-24 (1.3)
Hα-7	H-6 (2.2), Hβ-7 (2.3), H-10 (2.4)
Hβ-7	H-6 (1.7), Hα-7 (6.5), H-8 (2.2)
H-8	H_3-25 (3.4)
H-10	Hα-2 (3.3), H-3 (1.1), Hα-7 (2.3), Hα-12 (4.6), H_3-23 (1.3), H_3-26 (1.1)
Hα-12	H-10 (2.9), Hβ-12 (3.4)
H-18	H_3-26 (1.5), H_3-28 (1.9)
H-19	H_3-29 (1.2)
H-20	H_3-29 (3.4), H_3-30 (1.9)
H_3-23	Hα-2 (5.9), Hα-3 (6.7), H-10 (6.1), H_3-26 (1.0)
H_3-24	H-3 (5.7), H-6 (16.1), H_3-23 (1.1)
H_3-25	Hβ-7 (3.2)
H_3-26	H-6 (2.2), Hα-7 (3.8), H-10 (11.0), H-18 (13.8), H_3-23 (1.1)
H_3-27	Hβ-12 (5.2), Hβ-22 (3.1)
H_3-28	H-18 (20.7), Hα-22 (4.0), H_3-26 (1.0)
H_3-29[a]	H-20 (15.0)
H_3-30[a]	H-20 (30.0)

a) These are exchangeable.

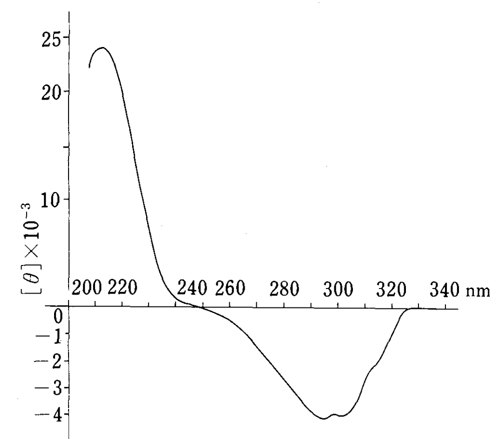

Fig. 1. CD Spectrum of **5**

An X-ray analysis proved the relative stereochemistry deduced above to be correct.[5] The ring-A and -B deformations, iso-Prα-19 and a ring-E puckered form with C(17) out of the plane were indicated. The ring-E conformation is affected by repulsion between iso-Pr-19 and H_3-28, causing deformation around C(17) in ring-D, and bending of H_3-28 toward H_3-26.[5] If it is assumed that the ring-D and -E solution conformations are similar to the solid-state ones, the mutual approaches between the two methyls account for an NOE observed between them.

To establish the absolute stereochemistry, we finally examined the CD spectrum of 3-dehydrohancokinol (**5**) which was easily obtained by oxidation of **1** with pyridinium dichromate. 3-Dehydrodesoxogratiogenin (**6**)[6] and alnusenone (**7**)[7] respectively reveal a negative Cotton effect at 295 nm (carbonyl n→π*) in the CD spectra. As depicted in Fig. 1, **5** exhibited a negative Cotton effect at 295 nm and the same curve at 325—249 nm as those of **6** and **7**. On the basis of the structural similarities of ring-A and -B, the 10*S*-configuration was assigned for **5**. Thus, the absolute stereochemistry of **1** was determined to be the 3*S*,8*R*,9*S*,10*S*,13*R*,14*S*,17*S*,18*S*,19*S*-configuration, *i.e.* 9β,13β-dimethyl-25,26-dinor-10α,17α-lup-5-en-3β-ol, on the basis of the relative stereochemistry.

Hancolupenone (**2**),[3] $C_{30}H_{48}O$, colorless plates, mp 241—242 °C (MeOH), $[\alpha]_D^{29}$ +14.4° ($CHCl_3$), showed an IR carbonyl band at 1702 cm^{-1}.

DEPT experiments showed the presence of eight methyls, nine methylenes, six methines and seven quaternary carbons. NMR analysis was carried out in the same manner as employed for **1**. The NMR parameters are given in Table III. ^1H–^1H COSY experiments indicated the presence of an isopropyl comprised to two *sec*-methyls δ_H 0.88 (H_3-29) and 0.90 (H_3-30) and a methine δ_H 1.53 (H-20). The ^1H–^1H and ^1H–^{13}C correlations of a methylene (δ_H 1.76, 1.12, δ_C 38.46, C-22) to a methylene (δ_H 1.76, 1.51, δ_C 28.90, C-21) and a methyl (δ_H 0.92, δ_C 32.60, C-28), which was related to a methine (δ_H 1.55, δ_C 54.17, C-18), suggested iso-Pr-19. A one-proton signal at δ_H 5.31 was attributed to a trisubstituted olefinic proton (H-11) possessing a neighboring methylene at δ_H 1.71 (H_2-12) (^1H–^1H). A carbonyl carbon at δ_C 217.34 (C-3) was correlated to a methylene at δ_H 2.40 (H-2) (^1H–^{13}C, long-range). A methine at δ_H 2.00 (H-8) was correlated to a neighboring methylene at δ_H 1.88 (H-7) (^1H–^1H).

A number of pentacyclic triterpenes with Δ^7, Δ^8 and Δ^9 unsaturations show characteristic mass spectrum (MS) fragments at m/z M^+-15 and M^+-167 and almost identical patterns for the remaining minor fragments.[4] As a result, these compounds cannot be distinguished from each other by mass spectroscopy. The electron impact mass spectra (EIMS) of **2** displayed a prominent fragment at m/z 206 arising from a retro-Diels–Alder fission in addition to fragments at m/z M^+-15 and M^+-167, suggesting a 13-methyl-26-norlup-9(11)-en-3-one for **2** by combining the NMR data.

The NOE data are shown in Table IV. The relative stereochemistry was examined assuming a chair form for each ring-A, -B and -D and a half-chair form for ring-C and using a basis of Meβ-10 (H_3-25). NOE experiments led to the following ring fusions: A/B *trans*, Hα (ax)-5 and Meβ (ax)-10 with respect to ring-A and -B; C/D *trans*, Meβ

2 : R = O

3 : R = OH, H

4 : R = $OCOC_{25}H_{51}(n)$, H

m/z M^+-167 retro D.-A. m/z 206

main NOEs

8 : R = O

10 : R = OMe, H

9 : R^1 = O, R^2 = Me

11 : R^1 = OAc, H, R^2 = —H

Chart 2

Table III. NMR Data for **2**

Carbon No.	δ_C	Correlated H[a] δ_H	H coupled with C[b]	H coupled with H[c]
1	36.50 t	Hα-1 1.80 dt (5.5, 13.5)	H_3-25	Hβ-1, H_2-2
		Hβ-1 2.05 ddd (13.5, 6.0, 3.0)		Hα-1, H_2-2
2	34.90 t	Hα-2 2.40 ddd (15.5, 5.5, 3.0)		H_2-1, Hβ-2
		Hβ-2 2.72 ddd (15.5, 13.5, 6.0)		H_2-1, Hα-2
3	217.34 s		Hα-2	
4	47.65 s		H-5, H_3-23, H_3-24	
5	53.39 d	H-5 1.36 dd (12.0, 3.0)		H_2-6
6	22.82 t	Hα-6 1.70 m	Hα-7	H-5, Hβ-6, Hβ-7
		Hβ-6 1.55 m		H-5, Hα-6, Hα-7
7	26.72 t	Hα-7 1.14 m	H-5, H_2-6, H-8	Hβ-6, Hβ-7
		Hβ-7 1.88 dq (12.0, 4.0)		Hα-6, Hα-7, H-8
8	42.25 d	H-8 2.00 br d (12.0)	H_3-27	Hβ-7
9	146.78 s		H_3-25	
10	39.18 s		H_3-25	
11	115.04 d	H-11 5.31 t (3.0)	H_2-12	H_2-12
12	36.95 t	H_2-12 1.71 d (3.0)	H_3-27	H-11
13	36.99 s		H-8, H-18, H-19, H_3-26, H_3-27	
14	37.34 s		H_3-26, H_3-27	
15	27.91 t	Hα-15 1.31 m	H_2-16, H_3-26	Hβ-15, H_2-16
		Hβ-15 1.44 m		Hα-15
16	32.23 t	H_2-16 1.45 m	Hα-22, H_3-28	Hα-15
17	41.16 s		H_3-28	
18	54.17 d	H-18 1.55 m	H_3-28	
19	49.53 d	H-19 1.49 m		Hβ-21
20	35.94 d	H-20 1.53 m	H-19, Hα-21	H_3-29, H_3-30
21	28.90 t	Hα-21 1.51 m	H-19, Hβ-22	Hβ-21, H_2-22
		Hβ-21 1.76 m		H-19, Hα-21, Hα-22
22	38.46 t	Hα-22 1.12 m	H-19, Hα-21, H_3-28	H_2-21, Hβ-22
		Hβ-22 1.76 m		Hα-21, Hα-22
23	25.55 q	H_3-23 1.07 s	H_3-24	
24	22.00 q	H_3-24 1.06 s	H-5, H_3-23	
25	21.30 q	H_3-25 1.20 s	Hα-1, H-5	
26	15.95 q	H_3-26 0.75 s		
27	15.67 q	H_3-27 0.74 s	H-18	
28	32.60 q	H_3-28 0.92 s	H_2-16, H-18, H-19, Hα-21	
29[d]	22.32 q	H_3-29 0.88 d (5.8)	H_3-30	H-20
30[d]	23.42 q	H_3-30 0.90 d (5.8)	H_3-29	H-20

a) ^{1}H–^{13}C (one-bond) COSY. Figures in parentheses are coupling constants (Hz). *b*) ^{1}H–^{13}C (long-range) COSY. *c*) ^{1}H–^{1}H COSY. *d*) These are exchangeable.

Table IV. NOE Data for **2**

Irradiated H	Observed H (%)
Hα-1	Hβ-1 (8.6), H-5 (2.6)
Hβ-2	Hα-2 (8.6)
Hα-7	Hα-6 (6.9), Hβ-7 (2.6)
Hβ-7	Hα-7 (2.6)
H-8	H_3-25 (1.0)
Hα-15	Hβ-15 (7.8)
Hα-22	Hβ-22 (6.9)
Hβ-22	Hα-22 (6.9)
H_3-23	Hα-1 (3.4), H-5 (10.3), Hα-6 (7.8)
H_3-24	Hβ-2 (2.6), H_3-25 (5.0)
H_3-25	Hβ-2 (3.4), Hβ-7 (5.2), H-8 (4.3), H_3-24 (4.0)
H_3-26	Hα-7 (5.2), H_2-12 (2.2), H-18 (8.6)
H_3-27	H-8 (5.6), Hβ-15 (4.3), H-19 (6.0), Hβ-21, Hβ-22 (5.2)
H_3-28	H_2-16 (2.8), H-18 (2.6), Hα-21 (11.2), H_3-26 (0.4)
H_3-29[a]	H-20 (2.2)
H_3-30[a]	H-18 (1.7), Hα-21 (22.1)

a) These are exchangeable.

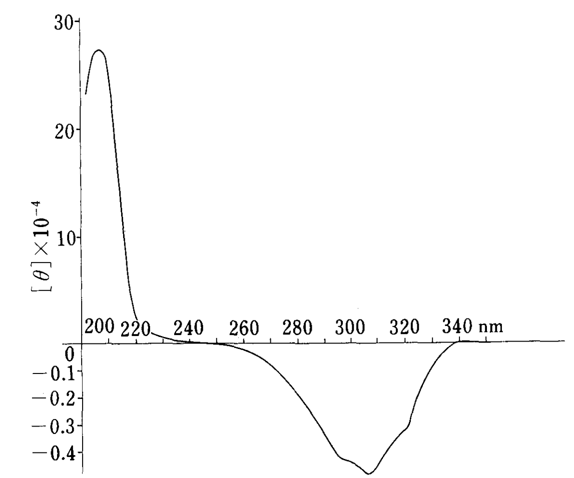

Fig. 2. CD Spectrum of **2**

(ax)-13 (H_3-27) and Meα (ax)-14 (H_3-26) with respect to ring-C and -D; D/E *cis*, Meα (eq)-17 (H_3-28) and Hα (ax)-18 with respect to ring-D. The enhancements linking to H_3-25 and H_3-27 suggested Hβ (ax)-8 with respect to ring- B and -C. An NOE observed between H-19 and H_3-27 led to iso-Prα-19.

A Dreiding model based on the NOE data showed the presence of steric hindrances in ring-A, -D and -E, probably causing the whole ring deformations. Such steric situations would be responsible for NOEs observed between Hα-1 (δ_H 1.80) and H_3-23, between Hβ-7 (δ_H 1.88) and H_3-25 and between H_3-26 and H_3-28.

An X-ray analysis proved the relative stereochemistry deduced above to be correct.[5] The ring-A, -B and -D deformations were found.

Arborinone (**8**) and lanost-9(11)-en-3-one (**9**) respectively show a negative Cotton effect in the carbonyl n→π* region in the optical rotatory dispersion (ORD) spectra.[8] Cylindrin (**10**), 4,4-dimethyl-5α,14β-cholest-9(11)-en-3β-ol acetate (**11**), **8** and analogous $\Delta^{9(11)}$-steroids respectively exhibit a positive Cotton effect in the olefinic π→π* region in the CD spectra.[9] On the other hand, arundoin (**10**: 8α,13α,14β,-17α,21α) indicates a negative Cotton effect.[9] A negative CD Cotton effect at 306 nm and a positive one at 201 nm observed for **2** (Fig. 2) led to the 10*S*- and 8*S*, 10*S*-configurations for **2**, respectively. Thus, the absolute stereochemistry was determined to be the 5*R*,8*S*,10*S*,13*R*,-14*S*,17*S*,18*S*,19*S*-configuration, *i.e.* 13β-methyl-26-nor-17α-lup-9(11)-en-3-one.

Hancolupenol (**3**),[3] $C_{30}H_{50}O$, colorless plates, mp 215—217 °C (MeOH), $[\alpha]_D^{29}$ +14.9° ($CHCl_3$), showed an IR hydroxyl band at 3602 cm^{-1}.

As shown in Table V,[10] NMR spectroscopy revealed that **2** and **3** possess the same framework with a difference in the 3-substituent. Sodium borohydride reduction of **2** gave **3**. Couplings (dd, J = 11.5, 4.0 Hz) observed for H-3 indicated HOβ (eq)-3, *i.e.* the 3*S*-configuration. The CD spectrum of **3** showed a positive Cotton effect at 203 nm, being in accord with the absolute stereochemistry of **2**.

TABLE V. NMR Data for **3**

Carbon No.	δ_C	Correlated H[a] δ_H	H coupled with H[b]
1	35.86 t	Hα-1 1.72 m	Hβ-1
		Hβ-1 1.62 ddd (11.0, 4.5, 2.5)	Hα-1
2	27.68 t	Hα-2 1.14 m	Hβ-2
		Hβ-2 1.69 m	Hα-2, H-3
3	78.96 d	H-3 3.22 dd (11.5, 4.0)	Hβ-2
4	39.06 s		
5	52.51 d	H-5 0.88 m	H_2-6
6	21.66 t	Hα-6 1.69 m	H-5, Hβ-6
		Hβ-6 1.44 m	H-5, Hα-6
7	27.14 t	Hα-7 1.12 m	H_2-6, Hβ-7, H-8
		Hβ-7 1.85 ddd (12.5, 8.0, 3.5)	H-6, Hα-7, H-8
8	42.17 d	H-8 1.94 br d (12.5)	H_2-7
9	148.25 s		
10	39.50 s		
11	113.71 d	H-11 5.25 dd (6.0, 3.0)	H_2-12
12	36.92 t	H_2-12 1.68 m	H-11
13	37.01 s		
14	37.31 s		
15	27.91 t	Hα-15 1.29 m	Hβ-15, H_2-16
		Hβ-15 1.38 m	Hα-15
16	32.65 t	H_2-16 1.44 m	Hα-15
17	41.19 s		
18	54.22 d	H-18 1.53 m	
19	49.53 d	H-19 1.50 m	Hβ-21
20	35.97 d	H-20 1.55 m	H_3-29, H_3-30
21	28.92 t	Hα-21 1.51 m	Hβ-21
		Hβ-21 1.74 m	H-19, Hα-21
22	38.50 t	Hα-22 1.16 m	Hβ-21, Hβ-22
		Hβ-22 1.74 m	Hα-22
23	28.18 q	H_3-23 0.98 s	
24	15.61 q	H_3-24 0.81 s	
25	21.83 q	H_3-25 1.01 s	
26	15.67 q	H_3-26 0.71 s	
27	16.01 q	H_3-27 0.74 s	
28	32.62 q	H_3-28 0.92 s	
29[c]	22.33 q	H_3-29 0.88 d (6.0)	H-20
30[c]	23.42 q	H_3-30 0.90 d (6.0)	H-20

a) ^{1}H–^{13}C (one-bond) COSY. Figures in parentheses are coupling constants (Hz). *b)* ^{1}H–^{1}H COSY. *c)* These are exchangeable.

Hancolupenol hexacosanoate (**4**), $C_{56}H_{100}O_2$, colorless needles, mp 99—101 °C ($CHCl_3$), $[\alpha]_D^{29}$ +18.0° ($CHCl_3$), showed an IR carboxyl band at 1720 cm^{-1}. ^{13}C-NMR spectroscopy indicated the presence of one methyl, a number of methylenes and one carboxyl carbon in addition to the carbon signals corresponding to **3**, suggesting **4** to be a fatty acid ester of **3** (H-3, δ_H 4.83). Hydrolysis of **4** gave **3** and hexacosanoic acid (**12**), each of which was identified with an authentic sample by direct comparison.

Experimental

General Experimental Procedures Melting points (uncorr.) were determined on a micro hot-stage apparatus. Specific rotations were taken on a JASCO DPI-181 polarimeter. Spectra were recorded on the following spectrometers: IR, Hitachi 260-30; ultraviolet (UV), Hitachi EPS-2U; CD, JASCO J-20; ^{1}H-NMR, Varian XL-400 at 400 MHz (reference, tetramethylsilane (TMS)); ^{13}C-NMR, Varian XL-400 at 100.6 MHz (reference, TMS); EIMS, Field desorption (FD) MS and fast atom bombardment (FAB) MS, JEOL JMS DX-300; elemental analysis, Perkin-Elmer 240B.

All NMR spectra were taken at a probe temperature of 20 °C in $CDCl_3$ using a 5 mm tube. The measuring conditions employed for **1**—**3** are shown. The values in parentheses are referred to **2** and **3**, being different from those of **1**.

The DEPT spectra were recorded using the $\theta = 90°$ and 142° pulses to separate the CH/CH_3 and CH_2 lines phased "up and down," respectively. Acquisition data were number of scans, 128—32000 (5120—32000, 25600—32000); relaxation delay for protons, 2 s; and 90° pulse widths, 31.0 μs and 9.6 μs for ^{1}H and ^{13}C, respectively. The delay between pulses (3.57 ms) was set to 1/2J(CH), where J(CH) was taken to be 140 Hz.

^{1}H–^{1}H COSY was done with a ^{1}H single probe; relaxation time 1 s; 90° (^{1}H) = 14.3 μs; 90° mixing pulse; $F_1 = F_2 = 712$ (1038, 2106) Hz; data matrix 512 × 64 (1024 × 128 for **3**): 126 (156, 64) scans during 64 (128 for **3**) time increments (zero filling in F_1); 2 dummy scans; spectra were symmetrized about a diagonal axis using the FOLDT command after 2D transformations.

^{1}H–^{13}C COSY was done under the following conditions: ^{13}C, 30—105 MHz probe; relaxation time, 1 s; 1 dummy scan; 90° ^{1}H and ^{13}C pulses were calibrated at 31.0 and 9.6 μs, respectively. One-bond correlation: $F_1 = 702$ (2048, 1907) Hz, $F_2 = 6618$ (10616, 10788) Hz; data matrix 1024 × 24 (2048 × 48 for **2** and **3**); 320 (704, 2560) scans during 64 (48 for **2** and **3**) time increments; acquisition time 0.077 (0.096, 0.095) s; $^1J_{CH}$ (average) 140 Hz; size of final data points, 2000. Long-range correlation: $F_1 = 2071$ (2048) Hz, $F_2 = 13351$ (13793) Hz; data matrix 2048 × 64; 704 (3712) scans during 64 (48) time increments (zero filling in F_1); 1 dummy scan; acquisition time 0.077 (0.074) s; $^{LR}J_{CH}$ (average) 7.0 Hz; size of final data points 2000.

The NOE spectra were recorded by means of NOE difference spectroscopy. The pre-irradiation time of each resonance was 2.25 s. The interpulse delay was 0 s. The irradiation data sets were interleaved to cancel drift and changing magnet homogeneity. These spectra were transformed by the difference between two free induction decays.

Extraction and Isolation The roots of *C. hancokianum* were collected in Inner Mongolia. Plant material was identified by Prof. Y. Guo, Shenyang College of Pharmacy, and a specimen is deposited in the Herbarium of Shenyang College of Pharmacy.

The air-dried, powdered plant material (15 kg) was extracted with boiling EtOH (30 l × 4) for several hours. MeOH–H_2O (6 : 4) (2.5 l) was added to the EtOH extract (2.3 kg), and the whole was extracted with petroleum ether (1.4 l × 5). A part (80%) of the whole petroleum ether extract (250 g) was chromatographed over silica gel (1.2 kg); elution was conducted successively with a mixture of petroleum ether and Me_2CO in 95 : 5 (solvent I), 90 : 10 (solvent II) and 85 : 15 ratio (solvent III). The product (2.2 g) from the solvent I eluate (1.5 l) was rechromatographed over silica gel (96 g) with petroleum ether–Me_2CO (97 : 3) (3 l) to yield **4** (90 mg). Further, the solvent I eluate (6.5 l) afforded **2** (150 mg). The solvent II eluate (1.5 l) gave **1** (1.3 g). Subsequent elution (1.5 l) yielded **3** (120 mg) on rechromatography of the crude eluate (2.8 g) over silica gel (100 g) eluting with solvent I (3.5 l). β-Sitosterol (2.4 g) was obtained by elution with solvent III (1 l). β-Sitosterol was identified with an authentic sample by direct comparison.

Hancokinol (1) Colorless needles, mp 229—230 °C (MeOH), *Rf* 0.45 (silica gel, $CHCl_3$). $[\alpha]_D^{20}$ +16.2° (c=0.77, $CHCl_3$), +16.6° (c=0.49, dioxane). IR ν ($CHCl_3$) cm^{-1}: 3580, 2936, 2866, 1449, 1376, 1136, 1106, 978. UV $\lambda_{max}^{dioxane}$ nm (ε): 278 (315). CD ($c = 8.7 \times 10^{-4}$, dioxane) $[\theta]^{20}$ (nm): 0 (228), +43100 (207) (positive maximum). EIHRMS *m/z*: M^+, 426.3862 (426.3862 for $C_{30}H_{50}O$). *Anal.* Calcd for $C_{30}H_{50}O$: C, 84.40; H, 11.81. Found: C, 84.17; H, 11.71.

3-Dehydrohancokinol (5) A mixture of **1** (5.0 mg) and pyridinium dichromate (31.0 mg) in dimethylformamide (0.06 ml) was stirred at room temperature overnight. The reaction mixture was diluted with H_2O and extracted with ether (5 ml × 3). Work-up of the organic layer, followed by preparative thin-layer chromatography (TLC) (silica gel, C_6H_6) of the product, gave **5** (4.5 mg, 90.5%) as colorless needles, mp 115—117 °C (MeOH), *Rf* 0.48 (silica gel, C_6H_6), $[\alpha]_D^{20}$ +17.2° (c=0.50, $CHCl_3$), +17.6° (c=0.50, dioxane). IR ν ($CHCl_3$) cm^{-1}: 2928, 2863, 1702, 1450, 1378, 1143, 1123, 1103, 963. UV $\lambda_{max}^{dioxane}$ (ε): 354 (257) (sh), 158 (300) (sh). CD ($c = 8.4 \times 10^{-4}$, dioxane) $[\theta]^{20}$ (nm): 0 (325), −2120 (314) (sh), −3990 (303) (negative maximum), −3940 (300) (positive maximum), −4100 (295) (negative maximum), −2120 (275) (sh), 0 (249), +24200 (212) (positive maximum). ^{13}C-NMR δ: 25.59 (t, C-1), 38.60 (t, C-2), 215.27 (s, C-3), 50.83 (s, C-4), 142.12 (s, C-5), 120.28 (d, C-6), 22.80 (t, C-7), 44.28 (d, C-8), 34.94 (s, C-9), 38.08 (d, C-10), 30.58 (t, C-11), 30.63 (t, C-12), 39.23 (s,

C-13), 39.79 (s, C-14), 29.42 (t, C-15), 32.62 (t, C-16), 39.93 (s, C-17), 54.49 (d, C-18), 48.25 (d, C-19), 35.94 (d, C-20), 27.84 (t, C-21), 42.54 (t, C-22), 27.98 (q, C-23), 22.54 (q, C-24), 28.66 (q, C-25), 15.24 (q, C-26), 16.86 (q, C-27), 33.38 (q, C-28), 21.27 (q, C-29), 23.43 (q, C-30). EIHRMS *m/z*: M^+, 424.3704 (424.3704 for $C_{30}H_{48}O$). *Anal.* Calcd for $C_{30}H_{48}O$: C, 84.84; H, 11.39. Found: C, 84.63; H, 11.38.

Hancolupenone (2) Colorless plates, mp 241—242 °C (MeOH), $[\alpha]_D^{29}$ +14.4° (c=0.20, $CHCl_3$), +16.7° (c=0.20, hexane). IR ν ($CHCl_3$) cm^{-1}: 2935, 2875, 1702, 1605, 1465, 1375. CD $[\theta]^{28}$ (nm): 0 (339), −328 (319) (sh), −480 (306) (negative maximum), −433 (296) (sh), 0 (245) ($c=1.45\times10^{-3}$, hexane), +27400 (207) (positive maximum) ($c=8.82\times10^{-4}$, hexane). EIHRMS *m/z*: M^+, 424.3698 (424.3707 for $C_{30}H_{48}O$). *Anal.* Calcd for $C_{30}H_{48}O$: C, 84.84; H, 11.39. Found: C, 84.67; H, 11.45.

Hancolupenol (3) Colorless plates, mp 215—217 °C (MeOH), $[\alpha]_D^{29}$ +14.9° (c=0.40, $CHCl_3$), +14.4° (c=0.40, hexane). IR ν ($CHCl_3$) cm^{-1}: 3602, 2927, 2909, 1599, 1457, 1374. CD ($c=1.78\times10^{-3}$, hexane) $[\theta]^{28}$ (nm): 0 (221), +27310 (203) (positive maximum). EIHRMS Calcd for $C_{30}H_{50}O$: M, 426.3860. Found *m/z*: M^+, 426.3846.

Reduction of 2 to 3 A solution of **2** (8.2 mg) and $NaBH_4$ (0.5 mg) in MeOH–tetrahydrofuran (1 : 1) (3 ml) was stirred at room temperature for 30 min. The reaction mixture was concentrated *in vacuo*, and the residue was extracted with ether (5 ml × 3). Work-up of the organic layer gave **3** (7.4 mg, 90%) as colorless plates, mp 215—217 °C (MeOH), which was identified with natural hancolupenol by direct comparison.

Hancolupenol Hexacosanoate (4) Colorless needles, mp 99—101 °C ($CHCl_3$), $[\alpha]_D^{29}$ +18.0° (c=0.10, $CHCl_3$). IR ν ($CHCl_3$) cm^{-1}: 2931, 2866, 1720, 1606, 1466, 1378. ^{13}C-NMR δ: 35.54 (t, C-1), 31.92 (t, C-2), 80.56 (d, C-3), 38.02 (s, C-4), 52.96 (d, C-5), 21.53 (t, C-6), 27.04 (t, C-7), 42.16 (d, C-8), 147.87 (s, C-9), 39.37 (s, C-10), 113.93 (d, C-11), 36.93 (t, C-12), 37.01 (s, C-13), 37.33 (s, C-14), 27.92 (t, C-15), 32.29 (t, C-16), 41.19 (s, C-17), 54.22 (d, C-18), 49.54 (d, C-19), 35.97 (d, C-20), 28.92 (t, C-21), 38.50 (t, C-22), 28.15 (q, C-23), 14.11 (q, C-24), 21.86 (q, C-25), 15.68 (q, C-26), 16.01 (q, C-27), 32.62 (q, C-28), 22.33 (q, C-29), 23.42 (q, C-30), 173.69 (s, CO), 34.86, 29.59, 25.17, 24.22, 22.69 (each t, CH_2), 29.70 (t, $CH_2\times n$), 16.83 (q, Me). ^{1}H-NMR δ: 4.83 (dd, J=12.0, 4.5 Hz, H-3), 2.30 (t, J=7.5 Hz, CH_2CO_2). FAB and FDMS *m/z*: $M^+ + 1$, 805 (804 for $C_{56}H_{100}O_2$).

Hydrolysis of 4 A mixture of **4** (2.7 mg), 2.5% KOH/MeOH (0.3 ml) and C_6H_6 (0.4 ml) was stirred at room temperature for 4 h. The reaction mixture was concentrated *in vacuo*, and the residue was extracted with C_6H_6 (2 ml × 2). Work-up of the organic layer afforded **3** (1.7 mg) as colorless plates, mp 215—217 °C (MeOH), which was identified with natural hancolupenol by direct comparison. The C_6H_6-insoluble portion was acidified with 5% HCl and evaporated to dryness, then extracted with AcOEt (2 ml × 2). Work-up of the organic layer gave **12** (1.0 mg) as colorless plates, mp 84—86 °C (AcOEt). EIHRMS Calcd for $C_{26}H_{52}O_2$: M, 396.3967. Found *m/z*: M^+, 396.3968. Compound **12** was identified with an authentic sample of haxacosanoic acid by direct comparison.

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Studies on Glycolipids. III.[1] Glyceroglycolipids from an Axenically Cultured Cyanobacterium, *Phormidium tenue*

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Seven new monogalactosyl diacylglycerols (1—7) and six new digalactosyl diacylglycerols (11—16) were isolated from an axenically cultured cyanobacterium, *P. tenue*. Their structures were elucidated on the basis of physicochemical evidence and the results of enzymatic hydrolysis using a lipase (from *Rhizopus arrhizus*). Comparison of antialgal activity for *P. tenue* between monogalactosyl diacylglycerols (1—8) and digalactosyl diacylglycerols (11—19) revealed that the former showed more potent activity than the latter.

Keywords axenic culture; cyanobacterium; *Phormidium tenue*; glycolipid; monogalactosyl diacylglycerol (MGDG); digalactosyl diacylglycerol (DGDG); antialgal activity; lipase; enzymatic hydrolysis

In a previous paper, we reported that fatty acids induced lysis of an axenically cultured cyanobacterium, *P. tenue*, and unsaturated fatty acids such as linoleic and linolenic acids showed higher antialgal activity (lysis inducing activity) than saturated ones.[2] Furthermore, we demonstrated that monogalactosyl diacylglycerols induced the lysis of this cyanobacterium, although their activities were less potent than those of fatty acids.[1] On the other hand, the cyanobacteria contain digalactosyl diacylglycerols as well as monogalactosyl diacylglycerols as major membrane lipids.[3] In this paper, we would like to present the full details of our studies on the structural elucidation of monogalactosyl diacylglycerols (MGDGs, **1**—**8**) and digalactosyl diacylglycerols (DGDGs, **11**—**19**), and their antialgal activity.

P. tenue was collected from the moat around the Nagoya Castle and an axenic culture was prepared by repeated capillary pipetting.[4] Mass culture was conducted in CT medium for 3 weeks in a laboratory. The alga was harvested by centrifugation and lyophilized. The lyophilized cells were extracted by the Bligh–Dyer method to obtain total lipid components.[5] The resulting extract was successively subjected to silica gel column chromatography to give MGDG and DGDG fractions in 1.3 and 0.9% yields, respectively. The MGDG fraction was further separated by reversed-phase high-performance liquid chromatography (HPLC) to furnish compounds **1**—**8** and a mixture of **9a** and **9b**.

Compound **1**, $[\alpha]_D^{24}$ $-2.7°$ ($CHCl_3$, $c=0.6$), gave a quasimolecular ion peak at m/z 747 $(M+Na)^+$ in its fast atom bombardment mass spectrum (FAB-MS). The infrared (IR) spectrum of **1** showed the presence of hydroxyl and ester groups, while the proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited two terminal methyl signals (δ 0.88, 0.96, 3H each, both t), a broad methylene signal at 1.26 ppm, and the signal (δ 2.41, 4H, m) due to two methylene protons linked to a carbonyl function. In addition, coupling constant analysis in the

1: R^1=linolenoyl; R^2=myristoyl
2: R^1=linolenoyl; R^2=palmitelaidoyl
3: R^1=linoleoyl; R^2=myristoyl
4: R^1=palmitoleoyl; R^2=myristoyl
5: R^1=oleoyl; R^2=myristoyl
6: R^1=linoleoyl; R^2=palmitoyl
7: R^1=palmitoyl; R^2=myristoyl
8: R^1=linolenoyl; R^2=linolenoyl
9a: R^1=linolenoyl; R^2=palmitoyl
9b: R^1=linoleoyl; R^2=palmitelaidoyl
(9a:9b=76:24)
10: R^1=R^2=H

11: R^1=linolenoyl; R^2=myristoyl
12: R^1=linoleoyl; R^2=myristoyl
13: R^1=palmitoleoyl; R^2=myristoyl
14: R^1=oleoyl; R^2=myristoyl
15: R^1=palmitoyl; R^2=myristoyl
16: R^1=linolenoyl; R^2=linoleoyl
17: R^1=linolenoyl; R^2=linolenoyl
18: R^1=linoleoyl; R^2=linoleoyl
19: R^1=linoleoyl; R^2=palmitoyl
20a: R^1=linolenoyl; R^2=palmitoyl
20b: R^1=linoleoyl; R^2=palmitelaidoyl
(20a:20b=72:28)

Chart 1

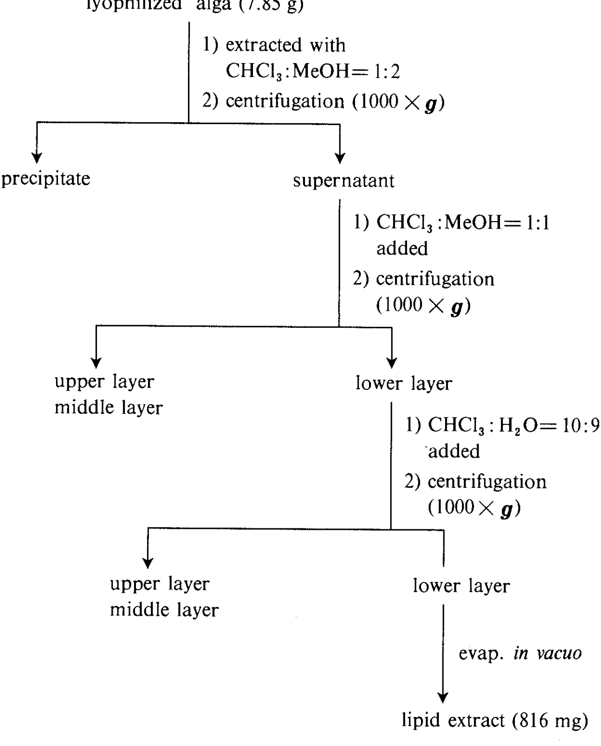

Chart 2

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1) NaOMe/MeOH
2) NaH, BnBr DMF
3) HCl–MeOH

MGDG (1—7)
DGDG (11—19)
21

Chart 3

lipase type XI (*Rhizopus arrhizus*)
Triton X-100
boric acid-borax buffer

1 : R^3=H
11 : R^3=α-D-galactopyranosyl
1a : R^3=H
11a : R^3=α-D-galactopyranosyl

Chart 4

homonuclear decoupling spectra defined a sugar component as a galactose; this was consistent with the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (Table I). Treatment of **1** with NaOMe–MeOH gave monogalactosyl-*sn*-glycerol (**10**),[6] methyl myristate and methyl linolenate. Detailed comparison of the ^{1}H-NMR spectrum of **1** with that of **10** disclosed that the proton signals due to 2-H and 1-H_2 in **1** were observed at a lower field than those in **10**.[7] Moreover, the ^{13}C-NMR spectra showed that the carbon signal due to C-1 of **10** appeared at a higher field than that of **1** and the carbon signals ascribable to C-2 and C-3 of both were observed at similar chemical shifts. On the basis of the above findings, it was concluded that the fatty acid residues were attached to C-1 and C-2 in the glycerol portion. The absolute configuration at C-2 in the glycerol portion of **1** was determined by Meguro's method.[8] Namely, the positive Cotton effect at 204 nm in the circular dichroism (CD) spectrum of 1,2-*O*-dibenzyl-*sn*-glycerol (**21**), which was prepared from **1** as illustrated in Chart 3, established 2*S*-configuration.

We next attempted regioselective deacylation of **1** under various conditions to determine the locations of the two fatty acid residues. On enzymatic hydrolysis using Lipase type XI from *Rhizopus arrhizus* in the presence of Triton X-100 in boric acid-borax buffer (pH 7.7), the galactolipid (**1**) liberated only linolenic acid to yield 2-monoacyl-3-*β*-D-galactopyranosyl-*sn*-glycerol (**1a**) quantitatively.[1b,9] The ^{1}H-NMR spectrum of **1a** showed the signals due to *sn*-1 methylene protons at higher field than those of **1**. The structure of **1a** was also confirmed by its ^{13}C-NMR spectrum; δ 68.8 (*sn*-1-C), 74.7 (*sn*-2-C), 61.7 (*sn*-3-C). Treatment of **1a** with NaOMe–MeOH afforded methyl myristate as a fatty acid component. Accordingly, the structure of the galactolipid (**1**) was determined as (2*S*)-1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-2-*O*-tetradecanoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol.

Compound **2**, $[\alpha]_D^{24}$ $-3.4°$ (c=0.4, $CHCl_3$), gave a quasimolecular ion peak at *m/z* 773 $(M+Na)^+$ in its FAB-MS. The ^{1}H-NMR spectrum of **2** closely resembled that of **1** except for the signals ascribable to the fatty acid residues. The lipase-catalyzed hydrolysis of **2** afforded linolenic acid and a 1-*O*-deacylated galactolipid, which gave a methyl ester on treatment with NaOMe–MeOH.

9 10 234 260 274 288 302 316 330

Fig. 1. Fragmentation Pattern of Picolinyl Ester of Palmitelaidic Acid

This methyl ester was found to have a molecular ion *m/z* 268 ($C_{17}H_{32}O_2$) by GC-MS analysis, but it was not methyl palmitoleate. So we applied the picolinyl method to clarify the location of its double bond.[10] Enzymatic hydrolysis and subsequent alkaline treatment (KOH–MeOH) of **2** gave an unidentified fatty acid, which was led to the corresponding acid chloride by thionyl chloride treatment. The acid chloride was converted to the picolinyl ester by using 3-pyridylcarbinol in CH_3CN. The fragmentation pattern in the GC-MS of the picolinyl ester revealed that the double bond was present between C-9 and C-10 (Fig. 1). The fatty acid, therefore, was determined to be palmitelaidic acid (9*E*-hexadecenoic acid) and the chemical structure of **2** was established as (2*S*)-1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-2-*O*-(9*E*-hexadecenoyl)-3-*β*-D-galactopyranosyl-*sn*-glycerol. With respect to other monogalactosyl diacylglycerols, the structures were elucidated in the same manner (Chart 1).

Separation of the DGDG fraction was performed by reversed-phase HPLC to furnish nine digalactosyl diacylglycerols (**11**—**19**) and a mixture of **20a** and **20b**. Compound **11**, $[\alpha]_D^{24}$ $+43.9°$ (c=0.8, MeOH), showed a substantial molecular ion peak at *m/z* 909 $(M+Na)^+$ in its FAB-MS. The IR spectrum of **10** was fairly similar to those of monogalactosyl diacylglycerols. Compound **11** showed close similarity to the galactolipid (**1**) in ^{1}H-NMR signals of the glycerol portion. Detailed analysis of the remaining signals in the ^{1}H- and ^{13}C-NMR spectra suggested that the galactolipid (**11**) possessed another galactose residue attached to the inner galactose unit. The α-configuration of the glycosidic bond between the two galactose residues was determined on the basis of the anomeric carbon signal at 100.7 ppm. The observation of the downfield shift of the C′-6 carbon confirmed a 1—6 linkage of the two galactose units. Deacylation of **11** with the lipase gave linolenic acid and a 1-*O*-deacylated galactolipid (**11a**), which was treated with NaOMe to afford methyl myristate. The stereochemistry of C-2 was defined to be *S* by the previous described procedure. On the basis of the above findings, the structure of **11** was clarified to be (2*S*)-1-*O*-(9*Z*,12*Z*,15*Z*-octadecatrienoyl)-2-*O*-tetradecanoyl-3-*O*-[α-D-galactopyranosyl(1—6)-*β*-D-galactopyranosyl]-*sn*-glycerol. The chemical structures of other digalactosyl diacylglycerols (**12**—**19**) were similarly elucidated, as illustrated in Chart 1.

We finally examined the antialgal activities of the monogalactosyl diacylglycerols (**1**—**8**) and the digalactosyl diacylglycerols (**11**—**19**) for *P. tenue*. The antialgal activity was determined by measuring chlorophyll a as described by Parsons and Strickland.[11] Relative growth rate of the alga was expressed as a percentage of the control, and IC_{50} of the test compounds was determined from dose-response curves. The concentrations (ppm) of the test compounds

TABLE I. ^{13}C-NMR Data[a] for **1**, **1a**, **10**, **11** and **11a**

Carbon	1	1a	10	11	11a
sn-1	64.0	68.8	64.1	64.0	68.9
sn-2	71.8	74.7	72.2	71.8	74.6
sn-3	68.7	61.7	72.1	68.8	61.8
1′	105.4	105.3	105.3	105.3	105.2
2′	72.4	72.5	72.6	72.6	72.5
3′	74.9	74.9	74.9	74.7[b]	74.7[b]
4′	70.2	70.3	70.4	70.1	70.2
5′	76.8	76.8	76.8	74.6[b]	74.6[b]
6′	62.5	62.5	62.6	67.9	67.9
1″				100.7	100.6
2″				70.3	70.2
3″				71.5	71.5
4″				71.2	71.1
5″				72.5	72.5
6″				62.9	62.8

a) The spectra were measured in CD_3OD at 100 MHz. *b*) Assignments may be interchangeable.

TABLE II. Concentration of Each Galactolipid Giving 50% Inhibition of Growth of *P. tenue* (ppm)

MGDG	ppm	DGDG	ppm
1	26	**11**	>100
2	32	**12**	>100
3	45	**13**	>100
4	>100	**14**	>100
5	>100	**15**	>100
6	27	**16**	>100
7	>100	**17**	>100
8	18	**18**	>100
		19	>100

causing 50% inhibition of growth of the alga after 3 d are summarized in Table II. The activities of the galactolipids containing unsaturated fatty acid residues were more potent than those of the galactolipids having saturated fatty acid residues in the case of the monogalactosyl diacylglycerols. In contrast, the digalactosyl diacylglycerols (**11**—**19**) exhibited no lysis-inducing activity regardless of the fatty acid residues.

In conclusion, we characterized seven new MGDGs (**1**—**7**) and six new DGDGs (**11**—**16**), and isolated three DGDGs (**17**—**19**)[12] as well as an identified galactolipid (**8**).[13] An examination of the antialgal activities of the isolated glyceroglycolipids for *P. tenue* revealed that the monogalactosyl diacylglycerols, which are major membrane lipids, induced the lysis of this cyanobacterium. However, the digalactosyl diacylglycerols showed no antialgal activity. Previously, we reported that unsaturated fatty acids such as linoleic and linolenic acids exhibited more potent antialgal activity than saturated fatty acids.[2] This result suggests that the lysis-inducing activities of the MGDGs are due to the fatty acids liberated by esterase in the alga. It is noteworthy that the galactolipids containing myristic acid are rare, and this is the first report of isolation of galactolipids containing palmitelaidic acid, in spite of the wide distribution of glyceroglycolipids in the plant kingdom.[14]

Experimental

IR spectra were recorded on a JASCO IRA-2 spectrometer. ^1H- and ^{13}C-NMR spectra were obtained with a JEOL GSX-400 (400 MHz) spectrometer using tetramethylsilane as an internal standard. FAB-MS were determined with JEOL DX-300 and JEOL DX-303 spectrometers. Optical rotations were measured on a JASCO DIP-4 digital polarimeter. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-8A. The conditions for identification of methyl esters of fatty acids were as follows: column, ULBON HR-SS-10 (0.25 mm i.d. × 50 m, Shinwa Kako Co., Ltd.); column temperature, 150—220 °C, 3 °C/min; injection temperature, 250 °C; carrier gas, N_2, 2.2 kg/cm^2. For GC-MS analysis, a JEOL D-300 mass spectrometer interfaced to a Hewlett Packard 5710A gas chromatograph with a JMA 2000 data processing system was employed. The conditions for GC-MS measurement were as follows. Gas chromatography: column, Silicone OV-101 (0.25 mm i.d. × 50 m); injection temperature, 300 °C; column temperature, 250—280 °C, 3 °C/min; carrier gas, He, 1.0 ml/min. Mass spectrometry: ionizing energy, 70 eV; ion source temperature, 230 °C. HPLC was performed using a JASCO 880-PU pump equipped with a Shodex RI, SE-11 differential refractometer. Medium pressure chromatography (MPLC) was carried out on a C.I.G. column system (Kusano Scientific Co., Ltd.; pump KPW-20, UV detector KU-331) with a prepacked column (20 mm i.d. × 100 mm, octadecyl silica, 20 μm). Thin layer chromatography (TLC) was performed on Merck precoated Kieselgel $60F_{254}$, and spots were detected by illumination with an ultraviolet lamp, or by spraying 5% vanillin–70% $HClO_4$, 1% $Ce(SO_4)_2$–10% H_2SO_4 followed by heating. Column chromatography was performed on silica gel BW-200 or BW-300 (Fuji Davison Chemicals Co., Ltd.).

Culture Conditions *P. tenue* was isolated from a water sample collected from the moat around Nagoya Castle in 1981. Axenic cultures were prepared by the repeated capillary pipette washing method and have been maintained in CT medium adjusted to pH 8.0 at 25 °C with cool-white fluorescent illumination of 1000 lux. The alga was cultured in 5-l Erlenmeyer flasks containing CT medium, *viz.* in g/l $Ca(NO_3)_2 \cdot 4H_2O$ 0.15; KNO_3 0.1; β-Na_2 glycerophosphate 0.05; $MgSO_4 \cdot 7H_2O$ 0.04; minor element solution 0.1 ml/l; trace elements solution 0.1 ml/l. The minor elements solution was composed of, in g/l $FeCl_3 \cdot 6H_2O$ 0.196; $MnCl_2 \cdot 4H_2O$ 0.036; $ZnSO_4 \cdot 7H_2O$ 0.022; $CoCl_2 \cdot 6H_2O$ 0.004; $Na_2MnO_4 \cdot 2H_2O$ 0.0025; $Na_2EDTA \cdot 2H_2O$ 1.0. The trace element solution consisted of, in g/l, vitamin B_{12} 0.1; biotin 0.1; thiamine·HCl 10.0. The pH of the medium was adjusted to 8.0 with sodium hydroxide prior to autoclaving. Cultures were illuminated continuously at an incident intensity of 1000 lux with cool-white fluorescent lamps and vigorously aerated with sterilized air passed through a 0.2 μm membrane filter (Millipore, Mirex FG-50) at the rate of 0.5 l/min. After three weeks, the alga was harvested by centrifugation at 20000 × *g* from the combined 40-l culture and lyophilized. Yields of lyophilized cells were typically in the range of 0.2—0.22 g/l of culture.

Isolation The lyophilized alga (7.85 g) was homogenized in $CHCl_3$: MeOH = 1 : 2 (300 ml), then the whole mixture was stirred for 20 min. The mixture was separated into supernatant and precipitate by centrifugation (1000 × *g*) and the resulting precipitate was extracted with $CHCl_3$: MeOH = 1 : 2 (300 ml) twice by the same procedure. $CHCl_3$: MeOH = 1 : 1 (600 ml) was added to the supernatant and the mixture was centrifuged at 1000 × *g* for 15 min. The upper and middle layers were discarded, then MeOH : H_2O = 10 : 9 (300 ml) was added to the lower layer. The mixture was centrifuged at 1000 × *g* for 15 min and the upper and middle layers were discarded. The resulting lower layer was concentrated under reduced pressure to yield a lipid extract (816 mg). The extract was subjected to silica gel column chromatography using $CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase) as the eluent to afford a crude MGDG fraction. The column was then eluted with $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase) to yield a crude DGDG fraction. The crude MGDG fraction was applied to a column of Sephadex LH-20 using $CHCl_3$ as the eluent to furnish the pure MGDG fraction (101 mg). The MGDG fraction was purified by HPLC (Develosil ODS A-5, MeOH : acetone : H_2O = 60 : 40 : 5, 10 mm i.d. × 250 mm, Nomura Chemical Co., Ltd.) to furnish **1** (31.2 mg), **2** (7.2 mg), **3** (37.2 mg), **4** (6.4 mg), **5** (4.9 mg), **8** (6.0 mg), a mixture of **6** and **7** (4.8 mg), and a mixture of **9a** and **9b** (8.6 mg). The mixture of **6** and **7** was further separated by HPLC (Develosil ODS K-5, 7.2 mm i.d. × 250 mm, MeOH : acetone : H_2O = 60 : 40 : 5) to give **6** (2.0 mg) and **7** (2.8 mg). The crude DGDG fraction was subjected to MPLC (MeOH : H_2O = 50 : 1) to afford pure DGDG fraction (70 mg). The fraction was purified by HPLC (Develosil ODS-5, 10 mm i.d. × 250 mm, MeOH : acetone : H_2O = 60 : 40 : 5) to furnish **11** (13.4 mg), **12** (27.2 mg), **13** (2.8 mg), **16** (4.8 mg), **17** (2.5 mg), **18** (2.9 mg), **19** (2.5 mg), a mixture of **14** and **15** (9.2 mg), and a mixture of **20a** and

20b (2.4 mg). The mixture of **14** and **15** were further separated by HPLC (Develosil ODS A-5, MeOH : CH_3CN : H_2O = 940 : 59 : 1, 10 mm i.d. × 250 mm) to yield **14** (5.1 mg) and **15** (4.0 mg). **1**: A colorless oil. $[\alpha]_D^{24}$ −2.7° (c=0.7, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 1730. FAB-MS *m/z*: 747 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.88 (3H, t, J=6.8 Hz), 0.96 (3H, t, J=7.5 Hz), 2.12 (4H, m), 2.41 (4H, m), 2.95 (4H, m), 4.08 (1H, dd, J=5.5, 5.7 Hz, 5′-H), 4.09 (1H, dd, J=5.0, 10.8 Hz, *sn*-3-H), 4.16 (1H, dd, J=3.4, 9.4 Hz, 3′-H), 4.40 (1H, dd, J=5.3, 10.8 Hz, *sn*-3-H), 4.42 (2H, m, 6′-H_2), 4.46 (1H, m, 2′-H), 4.54 (1H, dd, J=6.2, 10.8 Hz, *sn*-1-H), 4.57 (1H, d, J=3.4 Hz, 4′-H), 4.72 (1H, dd, J=3.1, 10.8 Hz, *sn*-1-H), 4.84 (1H, d, J=7.7 Hz), 5.42—5.46 (6H, m), 5.71 (1H, m, *sn*-2-H). Assignments for this compound were made with the aid of the homonuclear decoupling spectra. ^{13}C-NMR: Table I. **2**: A colorless oil. $[\alpha]_D^{24}$ −3.4° (c=0.8, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 1730. FAB-MS *m/z*: 773 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.88 (3H, t, J=6.8 Hz), 0.96 (3H, t, J=7.5 Hz), 2.09 (4H, m), 2.36 (2H, t, J=7.4 Hz), 2.49 (4H, br s), 2.94 (4H, m), 4.09 (1H, dd, J=5.7, 5.7 Hz, 5′-H), 4.10 (1H, dd, J=5.4, 10.9 Hz, *sn*-3-H), 4.16 (1H, dd, J=3.3, 9.3 Hz, 3′-H), 4.40 (1H, dd, J=4.8, 10.9 Hz, *sn*-3-H), 4.42 (2H, m, 6′-H_2), 4.46 (1H, m, 2′-H), 4.54 (1H, dd, J=3.3, 11.9 Hz, *sn*-1-H), 4.57 (1H, d, J=3.3 Hz, 4′-H), 4.70 (1H, dd, J=3.3, 11.9 Hz, *sn*-1-H), 4.84 (1H, d, J=7.7 Hz), 5.42—5.56 (8H, m), 5.70 (1H, m, *sn*-2-H). Assignments for this compound were made with the aid of the homonuclear decoupling spectra. **3**: A colorless oil. $[\alpha]_D^{24}$ −2.3° (c=0.8, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 1735. FAB-MS *m/z*: 749 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.87 (3H, t, J=6.8 Hz), 0.88 (3H, t, J=6.8 Hz), 2.12 (4H, m), 2.40 (4H, m), 2.93 (2H, dd, J=5.6, 5.8 Hz), 4.05—4.13 (2H, m, 5′-H, *sn*-3-H), 4.17 (1H, dd, J=3.4, 9.5 Hz, 3′-H), 4.40 (1H, dd, J=5.4, 11.0 Hz, *sn*-3-H), 4.43—4.50 (3H, m, 2′-H, 6′-H_2), 4.52—4.60 (2H, m, 4′-H, *sn*-1-H), 4.72 (1H, dd, J=3.4, 12.0 Hz, *sn*-1-H), 4.85 (1H, d, J=7.8 Hz, 1′-H), 5.52 (4H, m), 5.70 (1H, m, *sn*-2-H). Assignments for this compound were made with the aid of the homonuclear decoupling spectra. **4**: A colorless oil. $[\alpha]_D^{24}$ −2.8° (c=0.5, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3500, 1730. FAB-MS *m/z*: 723 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.88 (6H, m), 2.10 (4H, m), 2.39 (4H, m), 2.93 (2H, dd, J=5.6, 5.8 Hz), 4.06—4.12 (2H, m, 5′-H, *sn*-3-H), 4.17 (1H, dd, J=3.5, 9.4 Hz, 3′-H), 4.40 (1H, dd, J=5.4, 10.9 Hz, *sn*-3-H), 4.43—4.59 (2H, m, 4′-H, *sn*-1-H), 4.72 (1H, dd, J=3.3, 11.9 Hz, *sn*-1-H), 4.85 (1H, d, J=7.7 Hz, 1′-H), 5.52 (2H, m), 5.70 (1H, m, *sn*-2-H). **5**: A colorless oil. $[\alpha]_D^{24}$ −2.5° (c=0.5, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3520, 1730. FAB-MS *m/z*: 751 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.89 (6H, m), 2.12 (4H, m), 4.05—4.12 (2H, m, 5′-H, *sn*-3-H), 4.17 (1H, dd, J=3.3, 9.5 Hz, 3′-H), 4.40 (1H, dd, J=5.4, 10.8 Hz, *sn*-3-H), 4.42—4.50 (3H, m, 2′-H, 6′-H_2), 4.52—4.60 (2H, m, 4′-H, *sn*-1-H), 4.73 (1H, dd, J=3.1, 11.9 Hz, *sn*-1-H), 4.85 (1H, d, J=7.7 Hz, 1′-H), 5.51 (2H, m), 5.72 (1H, m, *sn*-2-H). **6**: A colorless oil. $[\alpha]_D^{24}$ −3.4° (c=0.6, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3530, 1735. FAB-MS *m/z*: 777 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.88 (6H, m), 2.12 (4H, m), 2.40 (4H, m), 2.93 (2H, t-like), 4.06—4.13 (2H, m, 5′-H, *sn*-3-H), 4.17 (1H, dd, J=3.1, 9.5 Hz, 3′-H), 4.40 (1H, dd, J=5.3, 10.8 Hz, *sn*-3-H), 4.43—4.51 (3H, m, 2′-H, 6′-H_2), 4.53—4.59 (2H, m, 4′-H, *sn*-1-H), 4.73 (1H, dd, J=3.1, 11.7 Hz, *sn*-1-H), 4.85 (1H, d, J=7.5 Hz, 1′-H), 5.51 (4H, m), 5.70 (1H, m, *sn*-2-H). **7**: A colorless oil. $[\alpha]_D^{24}$ −2.8° (c=0.5, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3530, 1735. FAB-MS *m/z*: 725 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 0.89 (6H, m), 2.39 (4H, m), 4.07—4.13 (2H, m, 5′-H, *sn*-3-H), 4.17 (1H, dd, J=3.3, 9.5 Hz, 3′-H), 4.40 (1H, dd, J=5.5, 11.2 Hz, *sn*-3-H), 4.43—4.52 (3H, m, 2′-H, 6′-H_2), 4.54—4.60 (2H, m, 4′-H, *sn*-1-H), 4.73 (1H, dd, J=3.1, 11.9 Hz, *sn*-1-H), 4.85 (1H, d, J=7.9 Hz, 1′-H), 5.70 (1H, m, *sn*-2-H). **11**: A colorless oil. $[\alpha]_D^{24}$ +43.9° (c=0.8, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1735. FAB-MS *m/z*: 909 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (3H, t, J=7.1 Hz), 0.97 (3H, t, J=7.5 Hz), 2.09 (4H, m), 2.32 (4H, m), 2.81 (4H, t, J=5.7 Hz), 3.49 (1H, dd, J=4.1, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=6.8, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.94 (1H, dd, J=5.5, 11.0 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.8, 12.1 Hz, *sn*-1-H), 4.24 (1H, d, J=6.8 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H), 5.30 (6H, m), ^{13}C-NMR: Table I. **12**: A colorless oil. $[\alpha]_D^{24}$ +44.6° (c=0.9, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1740. FAB-MS *m/z*: 911 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (3H, t, J=7.0 Hz), 0.91 (3H, t, J=7.0 Hz), 2.06 (4H, m), 2.32 (4H, m), 2.77 (2H, t, J=6.2 Hz), 3.48 (1H, dd, J=3.4, 9.8 Hz, 3′-H), 3.51 (1H, dd, J=6.6, 9.8 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.93 (4H, m), 3.93 (1H, dd, J=5.4, 10.8 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.6, 12.0 Hz, *sn*-1-H), 4.24 (1H, d, J=6.6 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.24 (1H, m, *sn*-2-H), 5.33 (4H, m). **13**: A colorless oil. $[\alpha]_D^{24}$ +42.1° (c=0.6, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1750. FAB-MS *m/z*: 885 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (6H, t, J=7.0 Hz), 2.06 (4H, m), 2.32 (4H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.3, 10.6 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.8, 12.0 Hz, *sn*-1-H), 4.24 (1H, d, J=7.0 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.0 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H), 5.35 (2H, m). **14**: A colorless oil. $[\alpha]_D^{24}$ +49.0° (c=0.6, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1725. FAB-MS *m/z*: 913 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.89 (6H, t, J=6.2 Hz), 2.03 (4H, m), 2.32 (4H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.3, 10.6 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.8, 12.1 Hz, *sn*-1-H), 4.24 (1H, d, J=7.0 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H), 5.34 (2H, m). **15**: A colorless oil. $[\alpha]_D^{24}$ +44.2° (c=0.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3410, 1725. FAB-MS *m/z*: 887 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (6H, t, J=6.6 Hz), 2.32 (4H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.5, 11.0 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.8, 12.1 Hz, *sn*-1-H), 4.24 (1H, d, J=7.0 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H). **16**: A colorless oil. $[\alpha]_D^{24}$ +46.1° (c=0.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3410, 1740. FAB-MS *m/z*: 961 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.91 (3H, t, J=7.0 Hz), 0.96 (3H, t, J=7.5 Hz), 2.08 (8H, m), 2.32 (4H, m), 2.80 (6H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.5, 11.0 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.8, 12.0 Hz, *sn*-1-H), 4.23 (1H, d, J=7.0 Hz, 1-H), 4.43 (1H, dd, J=2.9, 12.0 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H), 5.35 (10H, m). **17**: A colorless oil. $[\alpha]_D^{24}$ +33.7° (c=0.5, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3390, 1740. FAB-MS *m/z*: 959 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.91 (3H, t, J=7.3 Hz), 0.98 (3H, t, J=7.5 Hz), 2.07 (8H, m), 2.32 (4H, m), 2.81 (8H, m), 3.47 (1H, dd, J=3.0, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.5, 11.0 Hz, *sn*-3-H), 4.22 (1H, dd, J=7.0, 12.1 Hz, *sn*-1-H), 4.42 (1H, d, J=7.0 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.26 (1H, m, *sn*-2-H), 5.36 (12H, m). **18**: A colorless oil. $[\alpha]_D^{24}$ +51.2° (c=0.6, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1730. FAB-MS *m/z*: 963 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.91 (6H, t, J=6.8 Hz), 2.06 (8H, m), 2.34 (4H, m), 2.77 (4H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3-H), 3.51 (1H, dd, J=7.0, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.3, 10.6 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.7, 12.1 Hz, *sn*-1-H), 4.24 (1H, d, J=7.0 Hz, 1-H), 4.43 (1H, dd, J=2.9, 12.1 Hz, *sn*-1-H), 5.25 (1H, m, *sn*-2-H), 5.34 (8H, m). **19**: A colorless oil. $[\alpha]_D^{24}$ +47.6° (c=0.7, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3420, 1730. FAB-MS *m/z*: 939 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (6H, t, J=6.8 Hz), 2.06 (4H, m), 2.32 (4H, m), 2.78 (2H, m), 3.47 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.51 (1H, dd, J=7.1, 9.7 Hz, 2′-H), 3.63—3.80 (8H, m), 3.83—3.92 (4H, m), 3.93 (1H, dd, J=5.5, 11.0 Hz, *sn*-3-H), 4.22 (1H, dd, J=6.9, 11.9 Hz, *sn*-1-H), 4.24 (1H, d, J=7.1 Hz, 1′-H), 4.43 (1H, dd, J=2.9, 11.1 Hz, *sn*-1-H), 5.24 (1H, m, *sn*-2-H), 5.33 (4H, m).

Alkaline Treatment of 1 A solution of **1** (5.0 mg) in dry MeOH (1.0 ml) was treated with 5% NaOMe–MeOH (0.2 ml) at room temperature for 10 min. The reaction mixture was neutralized by using ion-exchange resin (Dowex 50 W × 8) and the resin was removed by filtration. The filtrate was extracted with hexane and the hexane layer was concentrated under reduced pressure to yield a mixture of methyl myristate and methyl linolenate (3.5 mg). The mixture of methyl esters was identified by GLC comparison with authentic samples. Removal of the solvent from the MeOH layer under reduced pressure gave a residue, which was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 6 : 4 : 1) to furnish **10** (1.7 mg). **10**: A colorless oil. $[\alpha]_D^{27}$ −8.0° (c=0.8, H_2O). IR ν_{max}^{KBr} cm^{-1}: 3500. FAB-MS *m/z*: 277 $(M+Na)^+$. ^{1}H-NMR (d_5-pyridine) δ: 4.08 (1H, dd, J=5.3, 6.6 Hz, 5′-H), 4.13 (1H, d, J=5.5 Hz, *sn*-1-H), 4.14 (1H, d, J=4.9 Hz, *sn*-1-H), 4.17 (1H, dd, J=3.3, 9.3 Hz, 3′-H), 4.27 (1H, dd, J=3.8, 9.7 Hz, *sn*-3-H), 4.45 (3H, m, 6′-H_2, *sn*-2-H, *sn*-3-H), 4.52 (1H, dd, J=7.7, 9.3 Hz, 2′-H), 4.56 (1H, d, J=3.3 Hz, 4′-H), 4.91 (1H, d, J=7.7 Hz, 1′-H). ^{13}C-NMR: Table I.

1,2-*O*-Dibenzyl-*sn*-glycerol (21) A solution of **1** (2.0 mg) in dry MeOH (1.0 ml) was treated with 5% NaOMe–MeOH (0.1 ml) at room temperature for 10 min. The reaction mixture was neutralized by using ion-exchange resin (Dowex 50 W × 8) and the resin was removed by filtration. The filtrate was concentrated under reduced pressure to give a product. A solution of it in dry DMF (0.5 ml) was treated with NaH (1.0 mg) at room temperature for 30 min. BnBr (6.4 μl) was added to the reaction solution, then the mixture was stirred at room temperature for a further 7.5 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl, and the mixture was poured into water. The whole was extracted with EtOAc, then the EtOAc extract was washed with saturated aqueous NaCl and dried over $MgSO_4$. Removal of the solvent under reduced

pressure gave a benzyl ether. A solution of it in 10% dry HCl–MeOH (1.0 ml) was heated under reflux for 3 h. The reaction mixture was poured into water and the whole was extracted with EtOAc. The EtOAc extract was washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a residue, which was purified by silica gel column chromatography (hexane : acetone = 5 : 1) to furnish 1,2-*O*-dibenzyl-*sn*-glycerol (**21**, 2.6 mg). **21**: A colorless oil. $[\alpha]_D^{25}$ −16.0° (c=0.3, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3410. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (1H, t-like, *sn*-3-OH), 3.58—3.78 (5H, m), 4.53—4.56 (2H, ABq, J=12.1 Hz, Ph–CH_2), 4.62—4.72 (2H, ABq, J=11.7 Hz, Ph–CH_2), 7.32 (10H, m, Ph–CH_2 × 2). UV λ_{max}^{MeOH} nm (ε): 212.5 (8800). CD (c=0.01, MeOH) $[\theta]^{23}$ (nm): $+1.3\times10^4$ (204) (positive maximum). MS m/z (%): 272 (M^+, 46). High resolution MS m/z: Calcd for $C_{17}H_{20}O_3$: 272.347, Found: 272.346.

Enzymatic Hydrolysis of 1 and 11 by the Use of Lipase (from *Rhizopus arrhizus*) A solution of **1** (5 mg) and Lipase type XI (700 unit) in the presence of Triton X-100 (2.5 mg) in boric acid–borax buffer (0.63 ml, pH 7.7) was stirred at 38 °C for 1 h. The reaction was quenched with acetic acid (0.1 ml), then EtOH was added to the reaction mixture. The solvent was removed under reduced pressure and the resulting residue was chromatographed on silica gel using $CHCl_3$–MeOH (7 : 1) as the eluent to yield **1a** (3.2 mg) and linolenic acid (1.8 mg). In the case of lipase-catalyzed hydrolysis of **11** (5.0 mg), **11a** (3.6 mg) was isolated by silica gel column chromatography with $CHCl_3$: MeOH : H_2O (7 : 3 : 1, lower phase) as the eluent. Enzymatic hydrolysis of the other MGDGs and DGDGs was carried out in the same manner. **1a**: A colorless oil. $[\alpha]_D^{26}$ −7.1° (c=0.7, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3425, 1730. FAB-MS m/z: 487 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (3H, t, J=6.8 Hz), 2.35 (2H, t, J=7.5 Hz), 3.45 (1H, dd, J=3.3, 9.7 Hz, 3′-H), 3.50 (1H, ddd-like, 5-H), 3.51 (1H, dd, J=7.3, 9.7 Hz, 2′-H), 3.66—3.79 (5H, m, 6′-H_2, *sn*-1-H_2, *sn*-3-H), 3.82 (1H, dd, J=0.9, 3.3 Hz, 4-H), 3.96 (1H, dd, J=5.7, 11.0 Hz, *sn*-3-H), 5.04 (1H, m, *sn*-2-H). ^{13}C-NMR: Table I. **11a**: A colorless oil. $[\alpha]_D^{26}$ +17.0° (c=0.4, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400, 1740. FAB-MS m/z: 649 $(M+Na)^+$. ^{1}H-NMR (CD_3OD) δ: 0.90 (3H, t, J=7.0 Hz), 2.35 (2H, m), 3.49 (2H, m, 2-H, 3-H), 3.63—3.80 (10H, m), 3.84—3.95 (5H, m), 4.24 (1H, d, J=7.3 Hz, 1-H). ^{13}C-NMR: Table I.

Identification of Palmitelaidic Acid by the Picolinyl Method Enzymatic hydrolysis of **2** by the lipase was carried out as above to yield 1-*O*-deacylmonogalactosylglycerol. A solution of it in dry MeOH (1.0 ml) was treated with 10% KOH–MeOH (1.0 ml) at room temperature for 10 min. Work-up of the reaction mixture as described above furnished palmitelaidic acid (0.6 mg). This fatty acid was treated with $SOCl_2$ (1.0 ml) at room temperature for 5 min. Excess $SOCl_2$ was removed, and the corresponding acid chloride in dry CH_3CN (0.1 ml) was treated with a 10% solution of 3-pyridylcarbinol in CH_3CN (0.1 ml) at room temperature for 10 min. Removal of the solvent by a stream of nitrogen gave a picolinyl ester, which was subjected to GC-MS analysis.

Assay of the Antialgal Activity Algal cultures for bioassay were grown to the late logarithmic growth phase for 10 d. A MeOH solution of each test compound (200 μl) sterilized through a membrane filter (Bio-Rad Inc., Micro Prep-disk, 0.2 μm pore size) was added to the CT-medium of the alga (20 ml). After incubation for 3 d, the cultures was filtered and chlorophyll a was extracted from the collected alga with 95% acetone. The concentration of chlorophyll a was determined photometrically as described by Strickland.[11] The IC_{50} of each galactolipid was determined from dose–response curves obtained with six different concentrations of test compounds (1, 5, 10, 25, 50, 100 ppm). The bioassay was carried out in duplicate.

Acknowledgement We thank Miss S. Kato of this Faculty for ^{1}H- and ^{13}C-NMR measurements. We are also greateful to Assoc. Prof. Y. Hotta and Mr. M. Naruse of Aichi Medical University for assistance with FAB-MS spectral measurements. We also wish to thank Mr. T. Ohki of the Bio-Dynamics Research Institute for running the GC-MS.

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Marine Natural Products. XXVII.[1] Distribution of Lanostane-Type Triterpene Oligoglycosides in Ten Kinds of Okinawan Sea Cucumbers

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Triterpene-oligoglycoside constituents in ten sea cucumber species inhabiting Okinawan coral reefs were investigated. Several antifungal oligoglycosides were isolated from six species [stichlorosides A_1 (15), A_2 (16), B_1 (17), B_2 (18), C_1 (19), and C_2 (20) from *Thelonota ananas* (baika-namako in Japanese) and *Stichopus hermanni*; 15, 17, and 19 from *Thelonota anax*; bivittosides C (13) and D (14) from *Bohadschia argus* (janome-namako); holothurin A (6) from *Holothuria edulis* (akamishikiri); and 6 and echinoside A (10) from *Bohadschia graeffei* (kurote-namako)]. Furthermore, several oligoglycosides were isolated in their desulfated forms from four species [6 and 10 from *Holothuria axiologa*; holothurin B (4), 6, echinoside B (8), and 10 from *Holothuria atra* (kuro-namako); 6, 10, and 24-dehydroechinoside A (24) from *Holothuria scabra* (haneji-namako); and 8, 10, 24-dehydroechinoside B (22), and 24 from *Actinopyga mauritiana* (kuriiro-namako)]. 24-Dehydroechinoside B (22) is a new lanostane-type triterpene oligoglycoside.

Keywords sea cucumber; lanostane-type triterpene oligoglycoside; saponin; holotoxin; holothurin; echinoside; bivittoside; stichloroside; 24-dehydroechinoside

In 1973, Elyakov *et al.* surveyed the chemical constituents of thirtyfour Pacific sea cucumber species collected in the islands of the West Pacific Ocean and discovered that triterpene saponins were widely distributed in Holothuroidea.[2] In 1974, we isolated two antifungal saponins named holotoxins A (**1**) and B (**2**), which exhibited significant antifungal activities, from the sea cucumber *Stichopus japonicus* (ma-namako in Japanese) and in 1978 determined the full structures of these lanostane-type triterpene oligoglycosides, in what was the first elucidation of the full structure of a sea cucumber saponin.[3]

Since then, we have searched for new antifungal saponins from various sea cucumbers.[4-10] We have so far isolated nineteen then-new lanostane-type triterpene oligoglycosides from various sea cucumbers and elucidated their chemical structures: holothurins A (**6**)[4a,b] and B (**4**)[5a,b] from *Holothuria leucospilota* BRANDT (nisekuro-namako), echinosides A (**10**) and B (**8**)[6a,b] from *Actinopyga echinites* JAEGER, bivittosides A (**11**), B (**12**), C (**13**), and D (**14**)[7a,b] from *Bohadschia bivittata* MITSUKURI (futasuji-namako), and stichlorosides A_1(**15**), A_2 (**16**), B_1 (**17**), B_2 (**18**), C_1 (**19**), and C_2 (**20**)[8a,b] from *Stichopus chloronotus* (BRANDT) (shikaku-namako). We have also isolated several new saponins in their desulfated forms and elucidated their chemical structures [holothurin A (**6**) and 24-dehydroechinoside A (**24**)[9] from the Bahamean sea cucumber *Actinopyga agassizi* SELENKA and pervicosides A (**25**), B (**26**), and C (**27**)[10] from *Holothuria pervicax* SELENKA (torafu-namako)].

In our continuing search for new bioactive marine natural products,[11] we have surveyed the distribution of oligoglycosidic constituents in ten more species of sea cucumbers collected in Okinawan coral reefs. In this paper, we present the details of our investigations on the saponin constituents of those sea cucumbers.[12]

In 1976, Kelecom *et al.* reported studies on the saponin constituent of the sea cucumber *Thelonota ananas*, which was collected at the coastal Indo-Pacific Ocean, and proposed partial structures of two saponins named thelothurins A and B.[13] On the other hand, we investigated the saponin constituent of the Okinawan sea cucumber of the same species (baika-namako in Japanese) which was collected at Zamami-jima, Okinawa Prefecture. The methanolic extract of the body walls of *T. ananas* was partitioned into a 1-butanol–water mixture and then the 1-butanol-soluble portion (=1-butanol extract) was subjected successively to centrifugal chromatography on silica gel and reversed-phase high-performance liquid chromatography (HPLC) to furnish stichlorosides A_1(**15**), A_2 (**16**), B_1 (**17**), B_2 (**18**), C_1(**19**), and C_2 (**20**), which were previously isolated from the sea cucumber *Stichopus chloronotus* by us,[8a,b] in 4, 2, 15, 8, 14, and 10% yields, respectively, from the 1-butanol extract.[12b]

In order to clarify the structural correlation of thelothurins A and B and our stichlorosides A_1, A_2, B_1, B_2, C_1, and C_2, we reinvestigated thelothurins A and B, the mixture of which was generously provided by Dr. Daloze (Université Libre de Bruxelles). It was found that the thelothurin mixture gave three spots on a silica gel thin-layer chromatogram (TLC) running with the same *Rf* values as those of stichlorosides A_1 (and A_2), B_1 (and B_2), and C_1 (and C_2).[8a,b] Thus, the thelothurin mixture was subjected successively to centrifugal chromatography on silica gel and reversed-phase HPLC to furnish six saponin constituents, which were found to be identical with authentic stichlorosides A_1 (**15**), A_2 (**16**), B_1 (**17**), B_2 (**18**), C_1 (**19**), and C_2 (**20**).[8a,b] Consequently, it has been concluded that the structures proposed for thelothurins A and B must be revised and the thelothurin mixture[14] comprises stichlorosides A_1 (**15**), A_2 (**16**), B_1 (**17**), B_2 (**18**), C_1 (**19**), and C_2 (**20**).

Further, two more sea cucumber species were shown to produce stichlorosides. Thus, stichlorosides A_1 (**15**), A_2 (**16**), B_1 (**17**), B_2 (**18**), C_1 (**19**), and C_2 (**20**) were isolated from the body wall of the sea cucumber *Stichopus hermanni* SEMPER, which was collected at Zamami-jima, Okinawa, in 0.6, 0.3, 1.7, 1.8, 8.6, and 3.5% yields, respectively from the 1-butanol-soluble portion of the methanolic extract of the body wall.[12b] In the case of the sea cucumber *Thelonota anax* H. L. CLARK, which was also collected at

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Chart 1

Chart 2

Zamami-jima, stichlorosides A_1 (**15**), B_1 (**17**), and C_1 (**19**) were obtained from the body wall in 7, 15, and 15% yields, respectively, from the 1-butanol-soluble portion.[12b)]

Several kinds of sea cucumbers belonging to *Bohadschia* sp., *Holothuria* sp., and *Actinopyga* sp. are equipped with Cuvierian tubules, defensive glands which extend from the anus when the sea cucumbers are attacked by other marine organisms. Cuvierian tubules contain a large amount of saponins which are piscicidal and seem to work as a chemical defence, especially against fish. It is noteworthy that the sea cucumber *Bohadschia graeffei* (SEMPER) (kurote-namako in Japanese) was found to contain echinoside A (**10**)[6b)] at an extremely high level in the body wall: it was isolated in 47% yield from the 1-butanol-soluble portion of the methanolic extract of the body wall, *i.e.* 0.9 g of echinoside A (**10**) from the body wall of one sea cucumber.[12b)] On the other hand, from the Cuvierian tubules of the same sea cucumber, holothurin A (**6**)[4b)] was isolated in 33% yield as a sole saponin from the 1-butanol-soluble portion of the methanolic extract of the Cuvierian tubules.[12b)] It is interesting that the Cuvierian tubules of this species contain a different kind of saponin from the saponin accumulated in the body wall.

In the case of the sea cucumber *Bohadschia argus* (JAEGER) (janome-namako in Japanese) which was collected at Kudaka-jima, Okinawa, bivittoside D (**14**)[7b] was obtained in 21% yield from the 1-butanol-soluble portion of the methanolic extract of the body wall, whereas bivittosides C (**13**)[7b] and D (**14**) were isolated from the 1-butanol-soluble portion of the Cuvierian tubules of the same sea cucumber in 7 and 37% yields, respectively.

Next, we investigated the saponin constituents of sea cucumbers belonging to *Holothuria* sp. and *Actinopyga* sp. The sea cucumber *Holothuria edulis* (LESSON) (akamishikiri in Japanese), collected at Okinawa Island, was shown to produce holothurin A (**6**),[4b] which was isolated in 21% yield from the 1-butanol-soluble portion of the body wall.[12a]

Silica gel column chromatography of the 1-butanol-soluble portion of the methanolic extract of the body wall of the sea cucumber *Holothuria scabra* JAEGER (hanejinamako) furnished an oligoglycoside mixture which showed a single spot on ordinary TLC. However, solvolysis[14] of this oligoglycoside mixture provided a mixture of three desulfated derivatives which were separated by reversed-phase HPLC to furnish desulfated holothurin A (**5**),[4b] desulfated echinoside A (**9**),[6b] and desulfated 24-dehydroechinoside A (**23**)[9] in 2, 10, and 3% yields, respectively from the 1-butanol-soluble portion. Thus, it has been concluded that the sea cucumber *H. scabra* produces holothurin A (**6**),[4b] echinoside A (**10**),[6b] and 24-dehydroechinoside A (**24**)[9] in an approximate 2 : 10 : 3 ratio.[12b]

From the sea cucumber *Actinopyga mauritiana* (QUOY & GAIMARD) (kuriiro-namako), which was collected at Kudaka-jima, Okinawa, two oligoglycosidic fractions, each giving a single spot on TLC, were obtained by silica gel column chromatography. Solvolytic degradation[14] of the polar oligoglycosidic fraction afforded a desulfated product which was separated by reversed-phase HPLC to furnish desulfated echinoside A (**9**)[6b] and desulfated 24-dehydroechinoside A (**23**)[9] in 6 and 2% yields, respectively from the 1-butanol-soluble portion. Similar solvolytic degradation of the less-polar oligoglycosidic fraction also yielded a desulfated product, which was separated by HPLC to furnish desulfated echinoside B (**7**)[6b] and a new desulfated oligoglycoside **21** in 1 and 1.5% yields, respectively, from the 1-butanol-soluble portion. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **21** showed two olefinic carbon signals [δ_C 124.8 (d), 131.6 (s)] attributable to the tri-substituted olefin at C-24 and C-25 and $\Delta^{9(11)}$ olefinic carbon signals which were comparable to those signals of desulfated echinoside B (**7**). Reduction of **21** with Raney Ni (W-4) furnished desulfated echinoside B (**7**) quantitatively, and thus the new desulfated oligoglycoside **21** has been confirmed to be a 24-dehydro analog of desulfated echinoside B (**7**). In conclusion, it has been clarified that the sea cucumber *A. mauritiana* produces echinosides A (**10**) and B (**8**), and 24-dehydroechinosides A (**24**) and B (**22**), in an approximate 6 : 1 : 2 : 1.5 ratio.[12a]

Then, we investigated the oligoglycoside constituents of *Holothuria axiologa* and *H. atra* using the same method as described above. The following results were obtained. *Holothuria axiologa* H. L. CLARK produces holothurin A (**6**) and echinoside A (**10**) in 12 : 7 ratio[12b] and *Holothuria atra* (JAEGER) (kuro-namako in Japanese) produces holothurins A (**6**) and B (**4**), and echinosides A (**10**) and B (**8**) in 7 : 5 : 7 : 5 ratio.[12a]

Chart 3

The location of the sulfate group in the oligoglycosidic constituent, which was produced by the sea cucumber *Holothuria scabra*, *Actinopyga mauritiana*, *H. axiologa*, or *H. atra* and furnished the respective desulfated oligoglycoside (*e.g.* **3**, **5**, **7**, **9**, **21**, or **23**) by solvolytic degradation, was determined by ^{13}C-NMR analysis of the sugar moiety to be at the 4-OH of the xylose moiety in each case.[4b,5b,9] Thus, both echinoside A (**10**) and 24-dehydroechinoside A (**24**) were shown to possess the same tetrasaccharide moiety including the location of the sulfate group (attached to the 4-OH of the xylose moiety) as that of holothurin A (**6**): the ^{13}C-NMR spectra of the sugar moieties in these oligoglycosides (**6**, **10**, **24**) were superimposable. Both echinoside B (**8**) and 24-dehydroechinoside B (**22**) were also shown to possess the same disaccharide moiety with the sulfate group attached to the 4-OH of the xylose moiety as in holothurin B (**4**), since here again the ^{13}C-NMR signals due to the sugar parts in these oligoglycosides (**4**, **8**, **22**)

TABLE I. Oligoglycoside (Lacking a Sulfate Group) Compositions of Sea Cucumbers

Sea cucumber		Oligoglycoside											
		Bivittoside				Holotoxin		Stichloroside					
		A	B	C	D	A	B	A_1	A_2	B_1	B_2	C_1	C_2
Bohadschia	Cuvierian tubules	+	+	+	+++								
bivittata	Body wall	+	+	+	+++								
Bohadschia	Cuvierian tubules			+	+++								
argus	Body wall				+++								
Stichopus japonicus						+	+						
Stichopus chloronotus								+	+	++	++	++	++
Stichopus hermanni								+	+	+	+	++	+
Thelonota ananas								+	+	++	++	++	++
Thelonota anax								+		++		++	

TABLE II. Oligoglycoside (Having a Sulfate Group) Compositions of Sea Cucumbers

Sea cucumber		Oligoglycoside								
		Holothurin		Echinoside		Δ^{24}-Echinoside		Pervicoside		
		A	B	A	B	A	B	A	B	C
Holothuria	Cuvierian tubules	+++	+							
leucospilota	Body wall		+++							
Holothuria atra		+++	+	+++	+					
Holothuria scabra		+		++		+				
Holothuria edulis		+++								
Holothuria axiologa		++		+						
Holothuria	Cuvierian tubules							+		
pervicax	Body wall							+	++	+
Actinopyga echinites				+++	+					
Actinopyga mauritiana				+++	+	++	+			
Actinopyga agassizi	(Cuvierian tubules)	+				+++				
Bohadschia	Cuvierian tubules	+++								
graeffei	Body wall			++++[a]						

a) Yield: 0.9 g from one fresh sea cucumber.

were superimposable. Furthermore, the ^{13}C-NMR signals due to the sugar moieties in components of the oligoglycoside mixture were observed as if they were the ^{13}C-NMR signals of a single compound, *i.e.* they were superimposable on those of holothurin A (**6**) or holothurin B (**4**).

The results obtained above and in our previous studies are summarized in Tables I and II. The sea cucumbers investigated by us are classified into two groups: the one comprises sea cucumbers of *Bohadschia* sp., *Stichopus* sp., and *Thelonota* sp., which produce triterpenic oligoglycosides lacking a sulfate group in their sugar moieties and the other contains sea cucumbers of *Holothuria* sp., *Actinopyga* sp., and *B. graeffei* which produce triterpenic oligoglycosides having a sulfate group attached to the 4-OH of the xylose moiety. Sea cucumbers of *Bohadschia* sp. were shown to contain both types.

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our previous paper.[15]

Oligoglycoside Constituents of the Sea Cucumber *Thelonota ananas* The fresh body walls (cut, 4.5 kg) of the sea cucumber *Thelonota ananas* (collected in August at Zamami-jima, Okinawa Prefecture) were extracted with MeOH (5 l each) under reflux 3 times for 4 h each. The MeOH extract (290 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water (4 l each) mixture to give the 1-butanol-soluble portion (49 g). The 1-butanol-soluble portion (2 g) was then purified by centrifugal chromatography [KT gel 2061 (Fuji-gel), $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to give three oligoglycoside fractions (fr. 1, 149 mg; fr. 2, 556 mg; fr. 3, 568 mg), which showed a single spot on TLC (silica gel, $CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower phase). These three oligoglycoside fractions were subjected to separation by HPLC (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to furnish stichlorosides A_1 (**15**) (76 mg) and A_2 (**16**) (42 mg) from fr. 1, stichlorosides B_1 (**17**) (306 mg) and B_2 (**18**) (167 mg) from fr. 2, and stichlorosides C_1 (**19**) (284 mg) and C_2 (**20**) (191 mg) from fr. 3. Stichloroside A_1 (**15**), mp 213—215 °C (MeOH), $[\alpha]_D$ −44° (pyridine, 19 °C). Stichloroside A_2 (**16**), mp 205—207 °C (MeOH), $[\alpha]_D$ −30° (pyridine, 19 °C). Stichloroside B_1 (**17**), mp 270—272 °C (MeOH), $[\alpha]_D$ −44° (pyridine, 19 °C). Stichloroside B_2 (**18**), mp 264—266 °C (MeOH), $[\alpha]_D$ −38° (pyridine, 19 °C). Stichloroside C_1 (**19**), mp 251—253 °C (MeOH), $[\alpha]_D$ −47° (pyridine, 19 °C). Stichloroside C_2 (**20**), mp 249—251 °C (MeOH), $[\alpha]_D$ −45° (pyridine, 19 °C). These oligoglycosides were identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[8a,b]

Purification of Thelothurin Mixture The thelothurin mixture (40 mg) provided by Dr. Daloze was purified by centrifugal chromatography [KT gel 2061 (Fuji-gel), $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to give three oligoglycoside fractions (fr. 1, 5 mg; fr. 2, 15 mg; fr. 3, 17 mg). Fr. 1, fr. 2, and fr. 3 were respectively shown to be mixtures of stichlorosides A_1 (**15**) and A_2 (**16**), stichlorosides B_1 (**17**) and B_2 (**18**), and stichlorosides C_1 (**19**) and C_2 (**20**) by HPLC comparison with authentic samples[8a,b] (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) and also by TLC comparison [Silica gel 60 F_{254} (Merck), $CHCl_3$: MeOH : H_2O = 65 : 35 : 10 (lower phase)]. Furthermore, stichloroside C_1 (**19**) (mp 251—253 °C), which was isolated from fr. 3 by HPLC, was shown to be identical with an authentic sample by mixed melting point determination.

Oligoglycoside Constituents of the Sea Cucumber *Thelonota anax* The

fresh body walls (cut, 3.5 kg) of the sea cucumber *Thelonota anax* (collected in August at Zamami-jima, Okinawa Prefecture) were extracted with MeOH (3 l each) under reflux 3 times for 4 h each. The MeOH extract (138 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water (2 l each) mixture to give the 1-butanol-soluble portion (15.7 g). The 1-butanol-soluble portion (500 mg) was then purified successively by centrifugal chromatography and HPLC as described above to furnish stichlorosides A_1 (**15**) (34 mg), mp 211—212 °C, $[\alpha]_D$ −44° (pyridine, 23 °C), B_1 (**17**) (77 mg), mp 269—271 °C, $[\alpha]_D$ −42° (pyridine, 23 °C), and C_1 (**19**) (77 mg), mp 255—256 °C, $[\alpha]_D$ −50° (pyridine, 23 °C). These oligoglycosides were identified by HPLC, mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[8a,b)]

Oligoglycoside Constituents of the Sea Cucumber *Stichopus hermanni* The fresh body walls (cut, 3.5 kg) of the sea cucumber *Stichopus hermanni* (collected in August at Zamami-jima, Okinawa Prefecture) were extracted with MeOH (4 l each) under reflux 3 times for 4 h each. The MeOH extract (45 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (7.5 g). The 1-butanol-soluble portion (5.6 g) was purified successively by column chromatography [Silica gel 60, 60—230 mesh (Merck), 400 g, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] and HPLC (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to furnish stichlorosides A_1 (**15**) [mp 211—213 °C, $[\alpha]_D$ −45° (pyridine, 19 °C)], 32 mg; A_2 (**16**) [mp 203—205 °C, $[\alpha]_D$ −34° (pyridine, 19 °C)], 18 mg; B_1 (**17**) [mp 268—269 °C, $[\alpha]_D$ −44° (pyridine, 19 °C)], 96 mg; B_2 (**18**) [mp 263—264 °C, $[\alpha]_D$ −37° (pyridine, 18 °C)], 104 mg; C_1 (**19**) [mp 250—252 °C, $[\alpha]_D$ −49° (pyridine, 20 °C)], 485 mg; and C_2 (**20**) [mp 248—250 °C, $[\alpha]_D$ −43° (pyridine, 18 °C)], 199 mg. These oligoglycosides were identified by HPLC, mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[8a,b)]

Oligoglycoside Constituents of the Sea Cucumber *Bohadschia graeffei* The fresh body walls (cut, 14 kg) of the sea cucumber *Bohadschia graeffei* (collected in August at Zamami-jima, Okinawa Prefecture) were extracted with MeOH (20 l each) under reflux 3 times for 4 h each. The MeOH extract (700 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (230 g). The 1-butanol-soluble portion (230 g) was treated with a $CHCl_3$ (400 ml)–MeOH (1500 ml)–H_2O (100 ml) mixture and the whole mixture was thoroughly stirred at room temperature (20 °C) for 24 h to yield a suspension. The insoluble portion (130 g) was then collected by filtration. Recrystallization of the insoluble portion (6 g) from $CHCl_3$: MeOH : H_2O furnished echinoside A (**10**) (5 g), which was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with an authentic sample.[6b)] The fresh Cuvierian tubules (0.9 kg) of the same sea cucumber were extracted with 95% aqueous MeOH (3 l) under reflux 3 times for 4 h each. The MeOH extract (69 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (25 g). The 1-butanol-soluble portion (25 g) was treated with a $CHCl_3$ (100 ml)–MeOH (600 ml)–H_2O (150 ml) mixture and the whole mixture was stirred at room temperature (25 °C) for 12 h. The insoluble portion (14 g) was collected by filtration. Recrystallization of the insoluble portion (3 g) from $CHCl_3$: MeOH : H_2O furnished holothurin A (**6**) (1.8 g), which was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with an authentic sample.[4b)]

Oligoglycoside Constituents of the Sea Cucumber *Bohadschia argus* The fresh body walls (cut, 27 kg) of the sea cucumber *Bohadschia argus* (collected in August at Kudaka-jima, Okinawa Prefecture) were extracted with MeOH (30 l each) under reflux 3 times for 4 h each. The MeOH extract, obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (120 g). The 1-butanol-soluble portion (10 g) was then purified by column chromatography [SiO_2 300 g, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to give bivittoside D (**14**) (2.1 g). Bivittoside D (**14**), mp 219—221 °C, $[\alpha]_D$ −7° (pyridine, 20 °C) was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with an authentic sample.[7b)]

The fresh Cuvierian tubules (1.4 kg) of the same sea cucumber were extracted with MeOH (2 l each) under reflux 5 times for 4 h each. The MeOH extract (130 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (65 g). The 1-butanol-soluble portion (32 g) was purified by column chromatography as described above to give bivittosides C (**13**), 2.3 g, mp 216—218 °C, $[\alpha]_D$ −31° (pyridine, 28 °C) and D (**14**), 12 g, mp 219—221 °C, $[\alpha]_D$ −7° (pyridine, 20 °C). These oligoglycosides were identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[7b)]

Oligoglycoside Constituents of the Sea Cucumber *Holothuria edulis* The fresh body walls (cut, 1.5 kg) of the sea cucumber *Holothuria edulis* (collected in August at Okinawa Island) were extracted with MeOH (1.5 l each) under reflux 3 times for 4 h each. The MeOH extract (31 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (5.7 g). The 1-butanol-soluble portion (5.7 g) was treated with $CHCl_3$-MeOH : H_2O (65 : 35 : 10, lower phase, 100 ml) and the whole mixture was stirred well at room temperature (20 °C) for 12 h. The insoluble portion (2.6 g) was collected by filtration and recrystallized from $CHCl_3$–MeOH–H_2O to furnish holothurin A (**6**) (1.2 g). Holothurin A (**6**) thus obtained [mp 228.5—230 °C, $[\alpha]_D$ −9° (pyridine, 24 °C)] was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with an authentic sample.[4b)]

Oligoglycoside Constituents of the Sea Cucumber *Holothuria scabra* The fresh body walls (cut, 4.3 kg) of the sea cucumber *Holothuria scabra* (collected in August at Okinawa Island) were extracted with MeOH (5 l each) under reflux 3 times for 4 h each. The MeOH extract (155 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (11 g). The 1-butanol-soluble portion (11 g) was then purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 1, lower phase) to give an oligoglycoside mixture (2.1 g). A solution of the oligoglycoside mixture (1.5 g) in dioxane (10 ml)–pyridine (20 ml) was heated under reflux for 1 h, then cooled. The residue, obtained after evaporation of the organic solvent under reduced pressure, was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 1, lower phase) to give a mixture of desulfated oligoglycosides (907 mg). The mixture (907 mg) was then subjected to HPLC separation (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to give desulfated holothurin A (**5**) (120 mg), mp 230—232 °C, $[\alpha]_D$ −3° (MeOH, 19 °C), desulfated 24-dehydroechinoside A (**23**) (185 mg), mp 242—244 °C, $[\alpha]_D$ −6° (pyridine, 20 °C), and desulfated echinoside A (**9**) (594 mg), mp 237—239 °C, $[\alpha]_D$ −1° (pyridine, 20 °C). The desulfated oligoglycosides thus obtained were identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[4b,6b,9)] The oligoglycoside mixture: ^{13}C-NMR (50 MHz, pyridine-d_5, δ_C): 105.6 (d, C-1′), 83.4 (d, C-2′), 77.6 (d, C-3′), 69.5 (d, C-4′), 64.1 (t, C-5′).

Oligoglycoside Constituents of the Sea Cucumber *Actinopyga mauritiana* The fresh body walls (cut, 13 kg) of the sea cucumber *Actinopyga mauritiana* (collected in August at Kudaka-jima) were extracted with MeOH (10 l each) under reflux 3 times for 4 h each. The MeOH extract (564 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (76 g). The 1-butanol-soluble portion (76 g) was treated with MeOH (1.3 l) and the whole mixture was stirred at room temperature (25 °C) for 12 h. The resulting suspension was then filtered to obtain the soluble portion (29 g) and the insoluble portion (45 g). The soluble portion (29 g) was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 1, lower phase) to give the oligoglycoside fraction I (3.8 g), which showed a single spot on TLC. The insoluble portion (45 g) was recrystallized from $CHCl_3$: MeOH : H_2O to give the oligoglycoside fraction II (12.1 g), which also showed a single spot on TLC.

A solution of the oligoglycoside fraction I (500 mg) in dioxane (8 ml)–pyridine (16 ml) was heated under reflux for 1.5 h, then cooled. The residue, obtained after evaporation of the organic solvent under reduced pressure, was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 10 : 3 : 1, lower phase) to give a mixture of desulfated oligoglycosides (240 mg). The combined desulfated oligoglycoside mixture (350 mg) was purified by HPLC (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 2 : 2 : 1) to furnish desulfated 24-dehydroechinoside B (**21**) (139 mg) and desulfated echinoside B (**7**) (100 mg). Desulfated 24-dehydroechinoside B (**21**), colorless needles, mp 239—240 °C, $[\alpha]_D$ +2° (pyridine, 18 °C). *Anal.* Calcd for $C_{41}H_{64}O_{13} \cdot 1/2H_2O$ = C, 63.66; H, 8.41. Found = C, 63.89; H, 8.69. ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 131.6 (s, C-25), 124.8 (d, C-24). Desulfated echinoside B (**7**), mp 245—246 °C, $[\alpha]_D$ +0.5° (pyridine, 24 °C) thus obtained was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with an authentic sample.[6b)]

A solution of the oligoglycoside fraction II (10 g) in dioxane (80 ml)–pyridine (160 ml) was heated under reflux for 1.5 h, then cooled. The residue, obtained after evaporation of the organic solvent under reduced pressure, was purified by SiO_2 column chromatography

[$CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] to give a mixture of desulfated oligoglycosides (6.5 g). The mixture of desulfated oligoglycosides (800 mg) was then subjected to HPLC purification (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to furnish desulfated 24-dehydroechinoside A (**23**) (152 mg), mp 243—244 °C, $[\alpha]_D$ −5° (pyridine, 18 °C) and desulfated echinoside A (**9**) (450 mg), mp 240—241 °C, $[\alpha]_D$ −1° (MeOH, 18 °C). These desulfated oligoglycosides were identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[6b,9)]

Oligoglycoside fraction I: ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 105.4 (d, C-1'), 83.0 (d, C-2'), 76.5 (d, C-3'), 75.0 (d, C-4'), 63.8 (t, C-5'). Oligoglycoside fraction II: ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 105.7 (d, C-1'), 83.1 (d, C-2'), 76.1 (d, C-3'), 74.9 (d, C-4'), 64.0 (t, C-5').

Reduction of Desulfated 24-Dehydroechinoside B (21) A solution of **21** (240 mg) in dry EtOH (70 ml) was treated with a suspension (40 ml) of Raney Ni (W-4)–EtOH and the whole mixture was heated under reflux for 14 h. After removal of the catalysts by filtration, the filtrate was evaporated under reduced pressure to give a product, which was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 10 : 3 : 1, lower phase) to furnish a reduction product (154 mg). The product thus obtained above was identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic desulfated echinoside B (**7**).[6b)]

Oligoglycoside Constituents of the Sea Cucumber *Holothuria axiologa* The fresh body walls (cut, 1.3 kg) of the sea cucumber *Holothuria axiologa* (collected in August at Zamami-jima, Okinawa Prefecture) were extracted with MeOH (3 l each) under reflux 3 times for 4 h each. The MeOH extract (65 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (18 g). The 1-butanol-soluble portion (16 g) was treated with MeOH (500 ml) and the whole mixture was stirred well at room temperature (20 °C) for 24 h. Filtration of the suspension gave the insoluble portion (6.1 g), which showed a single spot on TLC. The insoluble portion thus obtained (1 g) was treated with dioxane (20 ml)–pyridine (40 ml) and then the whole was heated under reflux for 1 h. The residue, obtained after evaporation of the organic solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (890 mg). The 1-butanol-soluble portion (394 mg) was subjected to HPLC purification (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to furnish desulfated holothurin A (**5**) (146 mg), mp 233—235 °C, $[\alpha]_D$ −2° (MeOH, 23 °C) and desulfated echinoside A (**9**) (79 mg), mp 235—237 °C, $[\alpha]_D$ −1° (pyridine, 23 °C). These desulfated oligoglycosides were identified by mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[4b,6b)] On the other hand, the above-mentioned MeOH-insoluble portion (1 g) was further purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 1, lower phase) to give an oligoglycoside fraction (800 mg). Oligoglycoside fraction: ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 105.5 (d, C-1'), 83.4 (d, C-2'), 77.6 (d, C-3'), 69.5 (d, C-4'), 64.1 (t, C-5').

Oligoglycoside Constituents of the Sea Cucumber *Holothuria atra* The fresh body walls (cut, 1.8 kg) of the sea cucumber *Holothuria atra* (collected in August at Okinawa Island) were extracted with 95% aqueous MeOH (1.5 l each) under reflux 4 times for 4 h each. The MeOH extract (78 g), obtained after removal of the solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (17 g). The 1-butanol-soluble portion was then treated with MeOH (340 ml) and the whole mixture was stirred well at room temperature (25 °C) for 12 h then filtered to give the MeOH-soluble portion (10 g) and the MeOH-insoluble portion (6.2 g). The MeOH-soluble portion was purified by SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 7 : 3 : 1, lower phase) to give the oligoglycoside fraction I (2.1 g), which showed a single spot on TLC ($CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower phase). The MeOH-insoluble portion was also subjected to SiO_2 column chromatography ($CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower phase) to give the oligoglycoside fraction II (4.0 g), which showed a single spot on TLC ($CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower phase).

A solution of oligoglycoside fraction I (980 mg) in dioxane (32 ml)–pyridine (16 ml) was heated under reflux for 1 h, then cooled. The residue, obtained after evaporation of the organic solvent under reduced pressure, was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (800 mg). The 1-butanol-soluble portion (130 mg) was then purified by HPLC (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 2 : 2 : 1) to furnish desulfated holothurin B (**3**) (58 mg), mp 275—278 °C, $[\alpha]_D$ −12° (MeOH, 19°C) and desulfated echinoside B (**7**) (56 mg), mp 258—260 °C, $[\alpha]_D$ +0.7° (pyridine, 19 °C). These desulfated oligoglycosides were identified by HPLC, mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[5b,6b)]

A solution of oligoglycoside fraction II (800 mg) in dioxane (30 ml)–pyridine (15 ml) was heated under reflux for 1 h, then cooled. The residue obtained as above was partitioned into a 1-butanol–water mixture to give the 1-butanol-soluble portion (630 mg). The 1-butanol-soluble portion (112 mg) was subjected to HPLC purification (μBondapak C_{18}, CH_3CN : MeOH : H_2O = 1 : 1 : 1) to furnish desulfated holothurin A (**5**) (39 mg), mp 231—233 °C, $[\alpha]_D$ −3° (MeOH, 19 °C) and desulfated echinoside A (**9**) (40 mg), mp 237—239 °C, $[\alpha]_D$ −1.2° (MeOH, 19 °C). These desulfated oligoglycosides were identified by HPLC, mixed melting point determination, and $[\alpha]_D$ and ^{13}C-NMR spectral comparisons with authentic samples.[4b,6b)] Oligoglycoside fraction I: ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 105.5 (d, C-1'), 82.9 (d, C-2'), 76.4 (d, C-3'), 74.8 (d, C-4'), 63.6 (t, C-5'). Oligoglycoside fraction II: ^{13}C-NMR (25 MHz, pyridine-d_5, δ_C): 105.7 (d, C-1'), 83.2 (d, C-2'), 76.2 (d, C-3'), 74.9 (d, C-4'), 64.0 (t, C-5').

Acknowledgement The authors are grateful to Prof. S. Ohta, Ocean Research Institute, University of Tokyo, for identification of the sea cucumber and to Prof. B. Tursch and Dr. D. Daloze of Universite Libre de Bruxelles for providing the thelothurin A and B mixture. This work was supported financially by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and a Grant-in-Aid from The Tokyo Biochemical Research Foundation.

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Synthesis and Anxiolytic Activity of *N*-Substituted Cyclic Imides (1*R**,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (Tandospirone) and Related Compounds[1,2]

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A series of cyclic imides bearing a ω-(4-aryl and 4-heteroaryl-1-piperazinyl)alkyl moieties was synthesized and tested *in vivo* for anxiolytic activity. The *in vitro* binding affinities of these compounds were also examined for 5-HT_{1A} receptor sites. Structure–activity relationships within these series are discussed. One of these compounds, (1*R,2*S**,-3*R**,4*S**)-*N*-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (1: tandospirone), was found to be equipotent with buspirone in its anxiolytic activity and more anxio-selective than buspirone and diazepam. Tandospirone (1) is currently undergoing clinical evaluation as a selective anxiolytic agent.**

Keywords bicyclo[2.2.1]heptanedicarboximide; tandospirone; anticonflict; 5-HT_{1A} receptor; structure–activity relationship; anxioselective anxiolytic activity; Mannich reaction

Buspirone is a new non-benzodiazepine anxiolytic agent[3] that demonstrates clinical efficacy comparable to that of diazepam without eliciting benzodiazepine-related side effects such as sedation, muscle relaxation, alcohol potenciation and abuse liability. However, buspirone was shown to have dopamine antagonistic activity as evidenced by its inhibition of apomorphine-induced stereotypy in rats.[4] In fact, buspirone was initially investigated as a potential antipsychotic agent, although a clinical study in acute schizophrenic patients failed to demonstrate definite antipsychotic activity. We hypothesized that the anti-dopaminergic activity of buspirone might not make any substantial contribution to its anxiolytic effects, and decided to search for analogues with more anxio-selective and less anti-dopaminergic activities than buspirone. At the time this work began, little attention was paid to modifications at the imide group, although a number of the analogues of buspirone had been synthesized for structure–activity studies.[5] Therefore, we investigated replacement of the 8-azaspiro[4,5]decane-7,9-dione group in buspirone with various imide moieties, and thus identified a compound, a 2,3-bicyclo[2.2.1]heptanedicarboximide derivative (1) which has desirable biological properties. This paper describes the synthesis and the biological activites of a series of imide-modified analogues related to buspirone,

Fig. 1

Chart 1

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in particular, focusing on the structure–activity relationships of various analogues of the 2,3-bicyclo[2.2.1]heptanedicarboximide (**1**).

Most of the *N*-substituted cyclic imides (I) listed in Tables I and II were prepared by modification of the methods of Wu and coworkers[5] from anhydrides (II) (method A) or imides (V) (method B) as outlined in Chart 1. Method A involved condensation of anhydrides (II) with ω-(4-aryl-1-piperazinyl)alkylamines (IV) prepared from the corresponding 1-arylpiperazines (VI). In method B, the imides (V) were converted to *N*-(ω-haloalkyl)imides (III), followed by reaction with VI. Compound **36** was prepared using method C in Chart 1, in which the *N*-[4-(1-piperazinyl)butyl]imide (**81**), prepared from the reaction of the corresponding *N*-(4-bromobutyl)imide (**75**) with 2-methylpiperazine, was converted to **36** by substitution with 2-chloropyrimidine.

The but-2-ynyl compounds (Ia)[6,7] were synthesized by the acetylenic Mannich reaction[8] outlined in Chart 2 (method D). Treatment of the *N*-propargylimides (VII), prepared from the imides (V), with the corresponding 1-arylpiperazines (VI) and aqueous formaldehyde in dioxane at 70—80 °C led to the Mannich product (Ia) in one step. The acetylene compounds (Ia) were easily converted to the corresponding saturated compounds (Ib)

V → VII (82−86) →(D) Ia (31, 54−62) →(E) Ib (1, 39, 46, 47, 52)

Chart 2

VIa → 87 →(68, F) 32

68 → 88 →(VIa, G) 33

Chart 3

1 →(H) 25; 26

Chart 4

TABLE I. Cyclic *N*-(4-Aryl-1-piperazinylalkyl)imide Derivatives

Compd. No.	R^1	B	N N	Ar	Yield (%)[a] (method)	mp (°C)	Formula	Analysis (%)[b] Calcd (Found) C	H	N
1		$(CH_2)_4$	$C_4H_8N_2$[c]	2-$C_4H_3N_2$[d]	81.3 (B) 59.0 (A) 88.6 (E)	170—171.5	$C_{21}H_{29}N_5O_2$ $\cdot C_6H_8O_7$[e]	56.34 (56.34	6.48 6.62	12.17 11.93)
2		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	70.6 (B) 91.4 (A)	115—116	$C_{21}H_{27}N_5O_2$	66.12 (66.08	7.13 7.43	18.36 17.92)
3		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	67.2 (A) 51.6 (B)	202—203	$C_{21}H_{29}N_5O_2$ $\cdot 2HCl$	55.26 (55.06	6.85 6.92	15.32 15.24)
4		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	62.3 (A)	210—213	$C_{20}H_{27}N_5O_3$ $\cdot 2HCl\cdot 1.2H_2O$	50.04 (50.16	6.59 6.62	14.59 14.56)
5		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	61.7 (A)	240—241	$C_{22}H_{31}N_5O_2$ $\cdot 2HCl$	56.17 (56.31	7.07 7.35	14.89 14.92)
6		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	68.1 (A)	206—210	$C_{20}H_{29}N_5O_2$ $\cdot 2HCl\cdot 0.5H_2O$	64.66 (64.70	7.87 7.79	18.85 19.05)
7		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	43.8 (A)	177—179	$C_{20}H_{27}N_5O_2$ $\cdot 2HCl\cdot 0.5H_2O$	53.22 (53.21	6.70 6.70	15.52 15.52)
8		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	69.2 (A)	156—158	$C_{21}H_{31}N_5O_2$ $\cdot 2HCl\cdot H_2O$	52.94 (52.58	7.40 7.28	14.70 14.96)
9		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	64.1 (A)	164—165.5	$C_{21}H_{29}N_5O_2$ $\cdot 2HCl\cdot 0.3H_2O$	54.61 (54.69	6.90 7.04	15.17 14.98)
10		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	62.4 (A)	225—227	$C_{22}H_{33}N_5O_2$ $\cdot 2HCl$	55.93 (55.70	7.47 7.64	14.82 14.73)
11		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	40.5 (A)	137 (dec.)	$C_{22}H_{33}N_5O_2$ $\cdot 2HCl\cdot 1.3H_2O$	53.29 (53.00	7.64 7.25	14.12 13.89)
12		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	37.1 (A)	208—210	$C_{22}H_{33}N_5O_2$ $\cdot 2HCl\cdot 0.3H_2O$	55.29 (55.66	7.51 7.83	14.66 14.27)
13		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	28.1 (A)	194—196	$C_{20}H_{27}N_5O_2$ $\cdot 2HCl\cdot 1.5H_2O$	51.17 (51.43	6.87 7.18	14.92 14.97)
14		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	85.6 (A)	140 (dec.)	$C_{32}H_{37}N_5O_2$ $\cdot 2HCl\cdot 0.5H_2O$ $\cdot$IPA[f]	63.15 (63.19	7.27 7.33	10.52 10.58)
15		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	86.1 (A)	212—215	$C_{18}H_{25}N_5O_2$ $\cdot 2HCl$	51.92 (51.92	6.54 6.56	17.03 16.96)
16		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	75.3 (A)	228—230	$C_{16}H_{23}N_5O_2$ $\cdot 2HCl$	49.23 (49.11	6.46 6.37	17.94 17.70)
17		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	37.5 (A)	230—234 (dec.)	$C_{22}H_{31}N_5O_2$ $\cdot 2HCl\cdot 0.4H_2O$	55.32 (55.29	7.13 7.22	14.67 14.70)
18		$(CH_2)_4$	$C_4H_8N_2$	2-$C_4H_3N_2$	82.6[g] 48.5 (A)	245—247	$C_{23}H_{33}N_5O_2$ $\cdot 2HCl$	57.02 (56.83	7.28 7.36	14.46 14.24)

TABLE I. (continued)

Compd. No.	R^1	B	N N	Ar	Yield (%)[a] (method)	mp (°C)	Formula	Analysis (%)[b] Calcd (Found) C	H	N
19		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	84.9 (A)	202—206	$C_{23}H_{31}N_5O_2 \cdot 2HCl \cdot 0.2H_2O$	56.83 (56.71	6.93 7.16	14.41 14.32)
20		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	84.3 (B) 29.0 (A)	138—139	$C_{20}H_{23}N_5O_2$	65.73 (65.79	6.34 6.31	19.17 19.03)
21		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	55.8 (A)	212.5—216	$C_{19}H_{22}N_6O_2$	62.28 (62.21	6.05 5.99	22.94 23.34)
22		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	80.3[h]	280—283	$C_{20}H_{24}N_6O_2 \cdot 2HCl$	52.98 (52.99	5.78 5.98	18.54 18.39)
23		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	23.0 (A)	227—230	$C_{16}H_{23}N_5O_3 \cdot 2HCl$	51.96 (51.51	6.54 6.61	18.94 18.47)
24		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	59.6 (A)	251—257	$C_{24}H_{25}N_5O_2 \cdot 2HCl \cdot 1.8H_2O$	55.34 (55.25	5.92 5.90	13.45 13.35)
25		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	72.9 (H)	144—147	$C_{21}H_{31}N_5O_2 \cdot 0.2H_2O$	64.82 (64.68	8.13 7.89	18.00 17.95)
26		$(CH_2)_4$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	68.9 (H)	294—296	$C_{21}H_{33}N_5 \cdot 3HCl \cdot 0.7H_2O$	52.82 (52.83	7.89 7.61	14.67 14.41)
27		$(CH_2)_3$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	81.6 (A) 84.9[i]	216—217	$C_{20}H_{27}N_5O_2 \cdot 2HCl \cdot H_2O$	52.17 (52.39	6.79 6.74	15.21 15.29)
28		$(CH_2)_5$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	67.7 (B)	139—146	$C_{22}H_{31}N_5O_2 \cdot C_6H_8O_7 \cdot H_2O$	55.35 (55.60	6.80 6.67	11.52 11.37)
29		$CH_2C(H){=}C(H)CH_2$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	86.8 (B)	150—152	$C_{21}H_{27}N_5O_2 \cdot C_6H_8O_7$	55.66 (55.81	6.23 6.15	12.02 12.12)
30		$CH_2C(H){=}C(H)CH_2$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	74.8 (B)	212—214	$C_{21}H_{27}N_5O_2 \cdot HCl \cdot 0.7H_2O$	58.58 (58.74	6.88 6.73	16.27 16.46)
31		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	99.3 (D)	122—123	$C_{21}H_{25}N_5O_2$	66.47 (66.07	6.64 6.64	18.46 18.29)
32		$CH_2CH(OH)CH_2CH_2$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	59.0 (F)	122—124.5	$C_{21}H_{29}N_5O_3$	63.13 (62.77	7.32 7.44	17.53 17.43)
33		$CH_2CH_2CH(OH)CH_2$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	82.2 (G)	119—120	$C_{21}H_{29}N_5O_3$	63.13 (62.89	7.32 7.45	17.53 17.44)
34		$(CH_2)_3CO$	$C_4H_8N_2$	$2\text{-}C_4H_3N_2$	78.4 (B)	185—188	$C_{21}H_{29}N_5O_3 \cdot HCl \cdot 0.2H_2O$	57.35 (57.70	6.97 6.97	15.93 15.93)
35		$(CH_2)_4$	$C_5H_{10}N_2$[j]	$2\text{-}C_4H_3N_2$	41.8 (B)	128 (dec.)	$C_{22}H_{31}N_5O_2 \cdot 2HCl \cdot 1.9H_2O$	52.36 (52.73	7.35 7.59	13.88 13.51)
36		$(CH_2)_4$	$2\text{-Me-}C_4H_7N_2$	$2\text{-}C_4H_3N_2$	44.8 (C)	116—117 (Base)	$C_{22}H_{31}N_5O_2 \cdot HCl$	61.03 (60.72	7.22 7.44	16.18 15.90)
37		$(CH_2)_4$	$3\text{-Me-}C_4H_7N_2$	$2\text{-}C_4H_3N_2$	57.9 (B)	177—180	$C_{22}H_{31}N_5O_2 \cdot 2HCl \cdot 2H_2O$	52.17 (52.51	7.36 7.23	13.83 13.54)
38		$(CH_2)_4$	$2,5\text{-Di(Me)-}C_4H_6N_2$	$2\text{-}C_4H_3N_2$	22.7 (B)	207—211	$C_{23}H_{33}N_5O_2 \cdot 2HCl \cdot 0.5H_2O$	55.92 (55.89	7.45 7.35	14.18 14.13)
39		$(CH_2)_4$	$C_4H_8N_2$	$5\text{-F-}2\text{-}C_4H_2N_2$	51.5 (B)	251—253	$C_{21}H_{28}FN_5O_2 \cdot HCl \cdot 0.2H_2O$	57.12 (57.09	6.71 6.54	15.86 15.93)
40		$(CH_2)_4$	$C_4H_8N_2$	$5\text{-Br-}2\text{-}C_4H_2N_2$	80.7 (B)	105—107	$C_{21}H_{28}BrN_5O_2$	54.55 (54.39	6.10 6.08	15.15 14.97)

TABLE I. (continued)

Compd. No.	R^1	B	N N	Ar	Yield (%)[a] (method)	mp (°C)	Formula	Analysis (%)[b] Calcd (Found) C	H	N
41		$(CH_2)_4$	$C_4H_8N_2$	4-Me- 2-$C_4H_2N_2$	62.5 (B)	103—104	$C_{22}H_{31}N_5O_2$	66.47 (66.32	7.86 7.93	17.62 17.53)
42		$(CH_2)_4$	$C_4H_8N_2$	4,6-Di(Me)- 2-C_4HN_2	56.6 (B)	248—250	$C_{23}H_{33}N_5O_2$ ·2HCl	57.02 (56.82	7.28 7.29	14.46 14.37)
43		$(CH_2)_4$	$C_4H_8N_2$	5-OH- 2-$C_4H_2N_2$	76.6[k] 52.3 (C)	198—200	$C_{21}H_{29}N_5O_3$	63.08 (63.05	7.32 7.29	17.53 17.48)
44		$(CH_2)_4$	$C_4H_8N_2$	5-$PhCH_2O$- 2-$C_4H_2N_2$	88.9 (B)	188—190	$C_{28}H_{35}N_5O_3$ ·2HCl·1.7H_2O	56.70 (56.62	6.87 6.59	11.81 11.73)
45		$(CH_2)_4$	$C_4H_8N_2$	4,6-Di(MeO)- 2-C_4HN_2	68.0 (B)	105—107	$C_{23}H_{33}N_5O_4$	62.28 (62.38	7.50 7.53	15.79 15.83)
46		$(CH_2)_4$	$C_4H_8N_2$	2-C_5H_4N[l]	25.2 (A)	150—152	$C_{22}H_{30}N_4O_2$ ·2HCl·H_2O	55.81 (55.81	7.24 7.23	11.83 11.47)
47		$(CH_2)_4$	$C_4H_8N_2$	3-CN- 2-C_5H_3N	76.8 (B)	180—184	$C_{23}H_{29}N_5O_2$ ·HCl	62.22 (62.01	6.81 6.83	15.78 15.67)
48		$(CH_2)_4$	$C_4H_8N_2$	3-Cl- 2-C_5H_3N	34.0 (B)	92—94	$C_{22}H_{29}ClN_4O_2$ ·2HCl·0.5H_2O	52.96 (52.91	6.47 6.41	11.23 11.16)
49		$(CH_2)_4$	$C_4H_8N_2$	5-Cl- 2-C_5H_3N	48.0 (B)	119—122	$C_{22}H_{29}ClN_4O_2$	63.37 (63.34	7.01 6.98	13.44 13.40)
50		$(CH_2)_4$	$C_4H_8N_2$	C_6H_5	51.4 (A)	229—231	$C_{23}H_{31}N_3O_3$ ·2HCl·0.2H_2O	60.34 (60.22	7.37 7.36	9.18 9.02)
51		$(CH_2)_4$	$C_4H_8N_2$	3-Cl-C_6H_4	55.7 (B)	218—220	$C_{23}H_{30}ClN_3O_2$ ·2HCl	56.50 (56.41	6.60 6.72	8.60 8.50)
52		$(CH_2)_4$	$C_4H_8N_2$	2-MeO- C_6H_4	90.5 (E)	92.5—93.5	$C_{24}H_{33}N_3O_3$	70.04 (69.97	8.08 8.11	10.21 10.20)
53		$(CH_2)_4$	$C_4H_8N_2$	3-CF_3- C_6H_4	55.6 (B)	210—212	$C_{24}H_{30}F_3N_3O_2$ ·2HCl	55.17 (55.24	6.17 6.23	8.08 7.92)
54		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	5-F- 2-$C_4H_2N_2$	75.5 (D)	202—203	$C_{21}H_{24}FN_5O_2$ ·HCl·1/3IPA	58.25 (58.27	6.07 6.11	15.44 15.33)
55		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	2-C_5H_4N	38.6 (D)	156—159	$C_{22}H_{26}N_4O_2$ ·HCl·1.1H_2O	60.78 (60.51	6.77 6.62	12.89 12.81)
56		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	3-CN- 2-C_5H_3N	51.8 (D)	185—189	$C_{24}H_{25}N_5O_2$ ·HCl·0.5H_2O	62.29 (61.91	5.92 5.98	15.13 15.51)
57		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	2-MeO- C_6H_4	Quant[m] (D)	169—170	$C_{24}H_{29}N_3O_3$ ·2HCl	60.00 (59.93	6.50 6.53	8.75 8.71)
58		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	2-$C_4H_3N_2$	81.0 (D)	109—110	$C_{21}H_{23}N_5O_2$	66.82 (66.74	6.14 6.11	18.56 18.48)
59		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	2-$C_4H_3N_2$	65.0 (D)	191—193	$C_{21}H_{27}N_5O_2$ ·HCl·0.3H_2O	59.58 (59.32	6.68 6.64	16.54 16.68)
60		$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	5-F- 2-$C_4H_2N_2$	67.6 (D)	194—196	$C_{21}H_{26}FN_5O_2$ ·HCl·0.2H_2O	57.38 (57.16	6.28 6.17	15.93 16.33)
61	Me Me	$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	2-$C_4H_3N_2$	95.5 (D)	201—202	$C_{19}H_{25}N_5O_2$ ·HCl·0.2H_2O	57.70 (57.73	6.73 6.66	17.71 17.74)
62	S—	$CH_2C{\equiv}CCH_2$	$C_4H_8N_2$	3-CN- 2-C_5H_3N	77.2 (D)	203—205	$C_{21}H_{23}N_5O_2S$ ·HCl	56.56 (56.34	5.42 5.47	15.70 15.55)

a) Yield of free base. *b)* Analytical results are within ±0.4% of the theoretical values in C, H, N analysis. *c)* $C_4H_8N_2$ represents piperazine. *d)* 2-$C_4H_3N_2$ represents pyrimidine. *e)* $C_6H_8O_7$ represents citric acid. *f)* IPA represents isopropyl alcohol. *g)* **18** was prepared by hydrogenation of **19** according to the similar method of D. *h)* **22** was prepared by cyclization of **107** (see Experimental section). *i)* **27** was prepared by hydrogenation of bicyclo[2.2.1]hept-5-ene-2,3-di-*exo*-carboximide derivative according to the similar method of D. *j)* $C_5H_{10}N_2$ represents homopiperazine. *k)* **43** was prepared by hydrogenolysis of **44** (see Experimental section). *l)* C_5H_4N represents pyridine. *m)* Quant represents quantitative yield.

TABLE II. Spectral Data of Cyclic Imide Derivatives

Compd. No.	Mass spectra m/z	IR spectra cm^{-1} (Nujol)	^{1}H-NMR spectra δ (ppm) ($CDCl_3$)[a,b]
1	383 (M+), 287, 275, 263, 220, 205, 177	1740, 1730, 1695, 1590, 1560[c]	8.30 (2H, d, J=5.1 Hz), 6.47 (1H, t, J=5.1 Hz), 3.82 (4H, t, J=5.1 Hz), 3.50 (2H, t, J=7.2 Hz), 2.70 (2H, s), 2.60 (2H, s), 2.46 (4H, t, J=5.1 Hz), 2.37 (2H, t, J=7.2 Hz), 1.66 (2H, d, J=6.1 Hz), 1.4—1.65 (4H, m), 1.32 (2H, d, J=6.1 Hz), 1.21 (1H, d, J=11.2 Hz), 1.09 (1H, d, J=11.2 Hz)
2	381 (M+), 286, 273, 261, 207, 177	1760, 1690, 1580, 1545, 1470, 1445	8.32 (2H, d, J=5.0 Hz), 6.48 (1H, t, J=5.0 Hz), 6.1—6.35 (2H, m), 3.84 (4H, t, J=5.1 Hz), 3.51 (2H, t, J=7.2 Hz), 2.25—2.8 (8H, m), 0.8—2.0 (6H, m)
3	383 (M+), 288, 275, 263, 220, 177	1760, 1700, 1690, 1585, 1545	8.30 (2H, d, J=4.9 Hz), 6.46 (1H, t, J=4.9 Hz), 3.83 (4H, t, J=4.9 Hz), 3.50 (2H, t, J=7.1 Hz), 2.1—3.2 (10H, m), 1.0—2.0 (10H, m)
4	385 (M+), 290, 277, 265, 177	1690, 1620, 1335	8.31 (2H, d, J=5.1 Hz), 6.48 (1H, t, J=5.1 Hz), 4.3—4.4 (2H, m), 3.83 (4H, t, J=5.1 Hz), 3.51 (2H, t, J=7.2 Hz), 2.80 (2H, s), 2.27—2.57 (6H, m), 1.37—1.97 (8H, m)
5	397 (M+), 302, 289, 277, 177	2870, 1700, 1585, 1550, 1495, 1445[c]	8.27 (2H, d, J=5.0 Hz), 6.46 (1H, t, J=5.0 Hz), 3.82 (4H, t, J=5.0 Hz), 3.49 (2H, t, J=7.0 Hz), 2.0—2.9 (10H, m), 1.1—1.9 (12H, m)
6	371 (M+), 276, 263, 251, 177, 121	2200—2800, 1760, 1700, 1640, 1610[c]	8.26 (2H, d, J=5.0 Hz), 6.45 (1H, t, J=5.0 Hz), 3.80 (4H, t, J=4.7 Hz), 3.46 (2H, t, J=7.0 Hz), 2.3—2.9 (8H, m), 1.1—2.0 (12H, m)
7	369 (M+), 274, 261, 249	2200—2800, 1760, 1685, 1630, 1410	8.30 (2H, d, J=5.0 Hz), 6.46 (1H, t, J=5.0 Hz), 5.8—5.95 (2H, m), 3.82 (4H, t, J=5.1 Hz), 3.50 (2H, m), 2.9—3.1 (2H, m), 2.0—2.8 (10H, m), 1.3—1.7 (4H, m)
8	385 (M+), 290, 277, 265, 177	2950, 1760, 1700, 1580, 1545, 1500[d]	8.28 (2H, d, J=5.0 Hz), 6.47 (1H, t, J=5.0 Hz), 3.82 (4H, t, J=4.9 Hz), 3.45 (2H, m), 0.67—3.23 (22H, m)
9	383 (M+), 288, 275, 263, 177	2930, 1760, 1700, 1580, 1540, 1500[d]	8.30 (2H, d, J=5.0 Hz), 6.45 (1H, t, J=5.0 Hz), 5.3—5.7 (1H, m), 3.88 (4H, t, J=5.0 Hz), 3.17—3.67 (2H, t, J=7.0 Hz), 2.1—3.2 (8H, m), 1.1—2.1 (11H, m)
10	399 (M+), 304, 291, 289, 177	1770, 1700, 1585, 1545, 1500, 1445[d]	8.37 (2H, d, J=5.0 Hz), 6.47 (1H, t, J=5.0 Hz), 3.82 (4H, t, J=4.9 Hz), 3.51 (2H, m), 2.3—2.9 (8H, m), 1.3—2.0 (10H, m), 0.67—1.0 (6H, m)
11	399 (M+), 304, 291, 279, 177	1770, 1700, 1590, 1550, 1500, 1400[d]	8.37 (2H, d, J=5.0 Hz), 6.44 (1H, t, J=5.0 Hz), 3.88 (4H, t, J=5.0 Hz), 3.3—3.7 (2H, m), 2.1—2.7 (8H, m), 0.9—2.1 (16H, m)
12	399 (M+), 304, 291, 279, 177, 123	1765, 1700, 1580, 1545, 1500, 1440[d]	8.32 (2H, d, J=5.0 Hz), 6.47 (1H, t, J=5.0 Hz), 3.80 (4H, t, J=5.0 Hz), 3.3—3.7 (2H, m), 2.3—2.6 (6H, m), 1.2—2.0 (12H, m), 1.17 (6H, s)
13	369 (M+), 274, 261, 249, 177	1760, 1700, 1580, 1545, 1450, 1400[d]	8.32 (2H, d, J=5.0 Hz), 6.47 (1H, t, J=5.0 Hz), 3.84 (4H, t, J=5.1 Hz), 3.4—3.7 (2H, m), 2.3—2.6 (6H, m), 0.9—2.1 (16H, m)
14	523 (M+), 427, 415, 403, 360, 177	1690, 1620, 1540, 1490, 1440, 1400[c]	8.12 (2H, d, J=5.0 Hz), 7.17 (10H, s), 6.27 (1H, t, J=5.0 Hz), 3.74 (4H, t, J=5.0 Hz), 2.8—3.6 (6H, m), 2.1—2.6 (10H, m), 1.3—2.0 (8H, m)
15	343 (M+), 248, 235, 223, 177, 121	1760, 1690, 1580, 1545, 1490, 1390	8.27 (2H, d, J=5.0 Hz), 6.43 (1H, t, J=5.0 Hz), 3.86 (4H, t, J=4.7 Hz), 3.60 (2H, t, J=7.0 Hz), 3.1—3.4 (2H, m), 2.2—2.9 (10H, m), 1.3—1.9 (4H, m)
16	317 (M+), 222, 209, 197, 177, 121	2200—2800, 1760, 1690, 1625, 1600	8.24 (2H, d, J=5.0 Hz), 6.43 (1H, t, J=5.0 Hz), 3.83 (4H, t, J=4.7 Hz), 3.52 (2H, t, J=7.0 Hz), 2.2—2.8 (10H, m), 1.4—1.8 (4H, m)
17	397 (M+), 302, 289, 277, 218, 205	2950, 1760, 1690, 1580, 1540, 1490	8.25 (2H, d, J=5.0 Hz), 6.40 (1H, t, J=5.0 Hz), 3.85 (4H, t, J=4.9 Hz), 3.60 (2H, t, J=7.0 Hz), 1.9—2.8 (12H, m), 0.9—1.9 (10H, m)
18	411 (M+), 330, 316, 303, 291	2200—2800, 1760, 1680, 1630, 1400	8.25 (2H, d, J=5.0 Hz), 6.40 (1H, t, J=5.0 Hz), 3.85 (4H, t, J=4.9 Hz), 3.60 (2H, t, J=7.0 Hz), 2.0—3.0 (8H, m), 1.0—2.0 (16H, m)
19	409 (M+), 330, 314, 301, 289	2200—2800, 1760, 1690, 1630, 1410	8.25 (2H, d, J=5.0 Hz), 6.30 (3H, m), 3.85 (4H, t, J=5.1 Hz), 3.50 (2H, m), 2.0—2.8 (10H, m), 1.0—2.0 (10H, m)
20	365 (M+), 270, 257, 245, 205, 177	2560, 1760, 1715, 1620	8.20 (2H, d, J=5.0 Hz), 7.5—7.8 (4H, m), 6.30 (1H, t, J=5.0 Hz), 3.80 (4H, t, J=4.9 Hz), 3.5 (2H, m), 2.2—2.5 (6H, m), 1.3—1.7 (4H, m)
21	366 (M+), 271, 258, 246, 205, 177	1770, 1710, 1580, 1545	8.8—9.0 (1H, m), 8.1—8.3 (3H, m), 7.5—7.7 (1H, m), 6.45 (1H, t, J=5.0 Hz), 3.85 (4H, t, J=4.9 Hz), 3.5 (2H, m), 2.2—2.7 (6H, m), 1.2—2.0 (4H, m)
22	380 (M+), 285, 272, 260, 218, 205	1710, 1655, 1595, 1545, 1500	8.23 (2H, d, J=4.5 Hz), 7.85—8.05 (1H, m), 7.4—7.7 (1H, m), 7.0—7.25 (2H, m), 6.50 (1H, t, J=4.5 Hz), 3.6—4.15 (6H, m), 2.3—2.6 (6H, m), 1.4—1.8 (4H, m)
23	333 (M+), 238, 225, 213, 177, 121	1730, 1680, 1580, 1555, 1500, 1400	8.27 (2H, d, J=5.0 Hz), 6.48 (1H, t, J=5.0 Hz), 4.32 (4H, s), 3.85 (4H, t, J=4.9 Hz), 3.5 (2H, m), 2.2—2.7 (6H, m), 1.3—1.8 (4H, m)
24	415 (M+), 320, 307, 295, 218, 205	2200—2800, 1700, 1655, 1600	8.50 (2H, d, J=8.7 Hz), 8.1—8.3 (4H, m), 7.5—7.8 (2H, m), 6.40 (1H, t, J=5.0 Hz), 4.0—4.3 (2H, m), 3.6—3.9 (4H, m), 2.3—2.6 (6H, m), 1.4—1.9 (4H, m)
25	385 (M+), 367, 290, 277, 247, 177	3300, 1650, 1585, 1550, 1480, 1440[c]	8.28 (2H, d, J=5.0 Hz), 6.47 (1H, t, J=5.0 Hz), 4.68 (1H, s), 3.80 (4H, t, J=4.9 Hz), 3.2—3.4 (2H, m), 2.25—2.55 (10H, m), 1.95 (1H, d, J=8.0 Hz), 1.65—1.8 (1H, m), 1.45—1.65 (5H, m), 1.15—1.3 (2H, m), 0.95—1.15 (2H, m)
26	355 (M+), 247, 235, 221, 179, 150	1585, 1545, 1485, 1470, 1445, 1360[d]	8.30 (2H, d, J=5.0 Hz), 6.48 (1H, t, J=5.0 Hz), 3.83 (4H, t, J=4.9 Hz), 2.95—3.25 (2H, m), 2.25—2.65 (8H, m), 1.75—2.25 (6H, m), 0.9—1.75 (10H, m)
27	369 (M+), 274, 261, 249, 121	1760, 1690, 1630, 1615	8.23 (2H, d, J=5.0 Hz), 6.43 (1H, t, J=5.0 Hz), 3.78 (4H, t, J=4.9 Hz), 3.55 (2H, t, J=7.0 Hz), 2.6—2.7 (2H, m), 2.58 (2H, s), 2.3—2.6 (6H, m), 1.05—1.9 (8H, m)
28	397 (M+), 302, 289, 277, 234, 177	1770, 1700, 1585, 1545, 1500, 1445[d]	8.32 (2H, d, J=5.0 Hz), 6.39 (1H, t, J=5.0 Hz), 3.87 (4H, t, J=4.9 Hz), 3.50 (2H, t, J=7.0 Hz), 2.7—2.8 (2H, m), 2.25—2.65 (8H, m), 1.0—1.9 (12H, m)
29	381 (M+), 286, 273, 261, 218, 203	3130, 1690, 1585, 1540, 1510, 1300	8.28 (2H, d, J=4.7 Hz), 6.43 (1H, t, J=4.7 Hz), 5.4—6.0 (2H, m), 4.05 (2H, d, J=4.5 Hz), 3.81 (4H, t, J=4.8 Hz), 2.98 (2H, d, J=5.4 Hz), 2.70 (2H, s), 2.62 (s), and 2.45 (t, J=4.8 Hz) 8H, 1.0—1.8 (6H, m)
30	381 (M+), 273, 203, 122, 108	2440, 1690, 1610, 1580, 1540, 1490	8.28 (2H, d, J=4.5 Hz), 6.43 (1H, t, J=4.5 Hz), 5.3—5.9 (2H, m), 4.10 (2H, d, J=6.0 Hz), 3.83 (4H, t, J=4.8 Hz), 3.21 (2H, d, J=6.0 Hz), 2.68 (s) and 2.61 (s) and 2.53 (t, J=4.8 Hz) 8H, 1.0—1.8 (6H, m)
31	379 (M+), 312, 271, 201, 122, 108	2430, 1760, 1710, 1580, 1555	8.33 (2H, d, J=4.5 Hz), 6.46 (1H, t, J=4.5 Hz), 4.25 (2H, t, J=2.3 Hz), 3.85 (4H, t, J=5.3 Hz), 3.30 (2H, t, J=2.3 Hz), 2.45—2.75 (8H, m), 1.1—1.8 (6H, m)
32	399 (M+), 291, 279, 177, 122	3140, 1760, 1690, 1580, 1550	8.28 (2H, d, J=4.8 Hz), 6.45 (1H, t, J=4.8 Hz), 3.3—3.9 (6H, m), 2.3—2.85 (10H, m), 1.1—1.8 (8H, m)

TABLE II. (continued)

Compd. No.	Mass spectra m/z	IR spectra cm^{-1} (Nujol)	^{1}H-NMR spectra δ (ppm) ($CDCl_3$)[a,b]
33	399 (M+), 381, 291, 279, 177, 148	3300, 1760, 1690, 1660, 1585, 1545	8.30 (2H, d, J=4.8 Hz), 6.48 (1H, t, J=4.8 Hz), 3.5—3.9 (7H, m), 3.0—4.0 (1H, br), 2.25—2.75 (10H, m), 3.05—3.8 (8H, m)
34	397 (M+), 382, 329, 277, 234, 219, 121	1760, 1695, 1640, 1580, 1550, 1490[d]	8.30 (2H, d, J=5.0 Hz), 6.52 (1H, t, J=5.0 Hz), 3.4—4.0 (10H, m), 2.5—2.75 (4H, m), 2.25—2.5 (2H, m), 0.95—2.15 (8H, m)
35	397 (M+), 382, 302, 289, 275, 261	1765, 1700, 1585, 1545, 1500, 1430[d]	8.35 (2H, d, J=5.0 Hz), 6.35 (1H, t, J=5.0 Hz), 3.65—4.0 (4H, m), 2.35—2.9 (10H, m), 1.75—2.3 (2H, m), 1.05—1.75 (10H, m)
36	397 (M+), 382, 302, 289, 277, 275, 261, 191	2370, 1765, 1695, 1590, 1560	8.30 (2H, d, J=5.4 Hz), 6.45 (1H, t, J=5.4 Hz), 4.82 (1H, m), 4.43 (1H, d, J=13.5 Hz), 3.51 (2H, t, J=7.3 Hz), 3.17 (1H, dt, J=3.1, 13.5 Hz), 2.87 (1H, d, J=11.6 Hz), 2.74 (1H, d, J=11.6 Hz), 2.70 (2H, s), 2.60 (2H, s), 2.33 (2H, m), 2.15 (1H, dd, J=3.9, 11.6 Hz), 2.00 (1H, dt, J=3.9, 11.6 Hz), 1.49 (2H, qi, J=7.3 Hz), 1.55—1.7 (4H, m), 1.32 (2H, d, J=6.9 Hz), 1.24 (3H, d, J=6.5 Hz), 1.19 (1H, d, J=9.6 Hz), 1.10 (1H, d, J=9.6 Hz)
37	397 (M+), 382, 289, 277, 191	2720, 1765, 1690, 1640, 1620	8.30 (2H, d, J=5.2 Hz), 6.46 (1H, t, J=5.2 Hz), 4.30 (2H, dt, J=3.1, 13.4 Hz), 3.46 (2H, t, J=7.3 Hz), 3.26 (1H, dt, J=3.1, 13.4 Hz), 2.95 (1H, dd, J=10.3, 13.4 Hz), 2.86 (1H, dt, J=3.5, 12.2 Hz), 2.75 (1H, dd, J=7.7, 13.5 Hz), 2.67 (2H, s), 2.60 (2H, s), 2.4 (1H, m), 2.2—2.35 (2H, m), 1.65 (2H, d, J=7.7 Hz), 1.54 (2H, qi, J=7.2 Hz), 1.45 (2H, qi, J=7.2 Hz), 1.32 (2H, d, J=7.7 Hz), 1.20 (1H, d, J=11.6 Hz), 1.07 (3H, d, J=6.9 Hz), 1.08 (1H, d, J=11.6 Hz)
38	411 (M+), 396, 303, 289, 277, 205	1765, 1700, 1585, 1545, 1490, 1440[d]	8.33 (2H, d, J=5.0 Hz), 6.43 (1H, t, J=5.0 Hz), 4.65—5.0 (1H, m), 4.39 (1H, dd, J=1.4, 13.2 Hz), 3.4—3.65 (3H, m), 2.25—3.3 (9H, m), 1.05—1.95 (13H, m), 0.98 (3H, d, J=6.6 Hz)
39	401 (M+), 275, 263, 195, 166	2480, 1770, 1700, 1620, 1565, 1480	8.19 (2H, s), 3.76 (4H, t, J=5.0 Hz), 3.49 (2H, t, J=7.0 Hz), 2.70 (2H, s), 2.60 (2H, s), 2.47 (4H, t, J=5.0 Hz), 2.38 (2H, t, J=7.0 Hz), 1.68 (2H, d, J=8.5 Hz), 1.5—1.65 (4H, m), 1.34 (2H, d, J=7.6 Hz), 1.22 (1H, d, J=11.0 Hz), 1.10 (1H, d, J=11.0 Hz)
40	463 (M+), 382, 288, 275, 263, 255	1760, 1685, 1570, 1520	8.28 (2H, s), 3.78 (4H, t, J=5.3 Hz), 3.49 (2H, t, J=6.7 Hz), 2.65—2.75 (2H, m), 2.60 (2H, s), 2.3—2.55 (6H, m), 1.1—1.75 (10H, m)
41	397 (M+), 288, 276, 263, 191, 122	1760, 1680, 1562, 1545, 1438, 790	8.12 (1H, d, J=4.9 Hz), 6.29 (1H, d, J=4.9 Hz), 3.78 (4H, t, J=5.0 Hz), 3.45 (4H, t, J=5.0 Hz), 2.55—2.7 (4H, m), 2.42 (6H, m), 2.29 (3H, s), 0.95—1.90 (10H, m)
42	411 (M+), 396, 288, 263, 205, 136	1760, 1700, 1570, 1500, 1440[d]	6.23 (1H, s), 3.82 (4H, t, J=5.2 Hz), 3.48 (2H, t, J=6.8 Hz), 2.70 (2H, s), 2.59 (2H, s), 2.35—2.6 (6H, m), 2.30 (6H, s), 1.0—1.8 (10H, m)
43	399 (M+), 288, 275, 263, 193	2210, 1755, 1690, 1595, 1580, 1545[d]	8.04 (2H, s), 3.72 (4H, t, J=5.0 Hz), 3.48 (2H, t, J=7.0 Hz), 2.59 (2H, s), 2.51 (2H, s), 2.45—2.55 (4H, m), 2.3—2.45 (2H, m), 2.45—2.75 (6H, m), 1.0—1.4 (4H, m)
44	489 (M+), 474, 398, 288, 275, 263	1770, 1695, 1550, 1480, 1445, 1400[c]	8.10 (2H, s), 7.25—7.4 (5H, m), 5.01 (2H, s), 3.65—3.75 (4H, m), 3.45 (2H, m), 2.96 (2H, s), 2.58 (2H, s), 2.46 (4H, t, J=3.8 Hz), 2.37 (2H, t, J=5.8 Hz), 1.66 (2H, d, J=2.8 Hz), 1.4—1.65 (4H, m), 1.32 (2H, d, J=2.8 Hz), 1.22 (1H, d, J=4.2 Hz), 1.08 (1H, d, J=4.2 Hz)
45	443 (M+), 288, 275, 263, 181, 168	1755, 1685, 1580, 1560, 1355, 1152	5.34 (1H, s), 3.87 (6H, s), 3.79 (4H, t, J=4.9 Hz), 3.48 (2H, t, J=5.1 Hz), 2.3—2.7 (10H, m), 1.0—1.7 (10H, m)
46	382 (M+), 288, 275, 263, 123, 120	1770, 1700, 1590, 1555, 1475, 1435[d]	8.05—8.2 (1H, m), 7.25—7.55 (1H, m), 6.45—6.65 (2H, m), 3.3—3.65 (6H, m), 2.3—2.75 (10H, m), 1.0—1.8 (10H, m)
47	407 (M+), 288, 275, 263, 201	2205, 1755, 1690, 1595, 1580, 1545[d]	8.33 (1H, dd, J=4.6, 1.8 Hz), 7.73 (1H, dd, J=7.4, 1.8 Hz), 6.70 (1H, dd, J=7.4, 4.6 Hz), 3.35—3.9 (6H, m), 2.15—2.75 (10H, m), 1.05—1.75 (10H, m)
48	416 (M+), 381, 288, 275, 263, 210	1770, 1690, 1575, 1540, 1435, 1400	8.17 (1H, dd, J=4.5, 2.0 Hz), 7.57 (1H, dd, J=7.5, 2.0 Hz), 6.78 (1H, dd, J=7.5, 4.5 Hz), 3.3—3.5 (6H, m), 2.3—2.7 (10H, m), 1.15—1.7 (10H, m)
49	416 (M+), 288, 275, 263, 210	1770, 1690, 1588, 1550, 1450, 1395	8.12 (1H, d, J=3.0 Hz), 7.42 (1H, dd, J=9.0, 3.0 Hz), 6.57 (1H, d, J=9.0 Hz), 3.45—3.55 (6H, m), 2.3—2.7 (10H, m), 1.2—1.7 (10H, m)
50	381 (M+), 275, 249, 175	1760, 1690, 1600, 1495, 1400, 1340	6.7—7.3 (5H, m), 3.3—3.65 (2H, m), 3.0—3.3 (4H, m), 2.2—2.85 (10H, m), 1.0—1.95 (10H, m)
51	415 (M+), 275, 249, 209, 166	1765, 1700, 1595, 1560, 1485, 1450[d]	6.95—7.2 (1H, m), 6.65—6.95 (2H, m), 3.35—3.6 (2H, m), 3.05—3.35 (4H, m), 2.25—2.75 (10H, m), 1.05—1.75 (10H, m)
52	411 (M+), 275, 205, 120	1770, 1695, 1598, 1500, 1240	6.7—7.05 (4H, m), 3.78 (3H, s), 3.35—3.6 (2H, m), 2.95—3.2 (4H, m), 2.5—2.75 (8H, m), 2.3—2.5 (2H, m), 1.0—1.8 (10H, m)
53	449 (M+), 275, 249, 243, 200	1765, 1700, 1600, 1585, 1495, 1450[d]	7.25—7.45 (1H, m), 6.95—7.25 (3H, m), 3.4—3.7 (2H, m), 3.2—3.4 (4H, m), 2.35—2.85 (10H, m), 1.05—1.85 (10H, m)
54	382 (M+), 330, 232, 219, 126	2320, 1760, 1700, 1603, 1550, 1490	8.23 (2H, s), 4.25 (2H, t, J=2.3 Hz), 3.82 (4H, t, J=4.8 Hz), 3.33 (2H, t, J=2.3 Hz), 2.75—2.45 (8H, m), 1.75—1.1 (6H, m)
55	378 (M+), 310, 271, 200, 119, 107	2300, 1760, 1690, 1630, 1600, 1585	8.15 (1H, dd, J=2.0, 4.8 Hz), 7.55—7.3 (1H, m), 6.65—6.50 (2H, m), 4.20 (2H, t, J=2.2 Hz), 3.56 (4H, t, J=4.8 Hz), 3.30 (2H, t, J=2.2 Hz), 2.75—2.50 (8H, m), 1.75—1.1 (6H, m)
56	403 (M+), 336, 271, 225, 119	2420, 1755, 1700, 1580, 1545, 1175	8.33 (1H, dd, J=2.4, 5.4 Hz), 7.74 (1H, dd, J=2.4, 7.8 Hz), 6.72 (1H, dd, J=5.4, 7.8 Hz), 4.22 (2H, t, J=1.8 Hz), 3.76 (4H, t, J=5.1 Hz), 3.32 (2H, t, J=1.8 Hz), 2.75—2.55 (8H, m), 1.90—1.10 (6H, m)
57	407 (M+), 271, 189, 136, 107	2350, 1755, 1690, 1600, 1585, 1490	7.1—6.7 (4H, m), 4.23 (2H, t, J=2.1 Hz), 3.83 (3H, s), 3.30 (2H, t, J=2.1 Hz), 3.2—3.0 (4H, m), 2.8—2.6 (8H, m), 1.8—1.5 (6H, m)
58	377 (M+), 311, 269, 201, 122, 108	1765, 1700, 1590, 1555, 1500	8.28 (2H, d, J=5.0 Hz), 6.45 (1H, t, J=5.0 Hz), 6.25—6.4 (2H, m), 4.29 (2H, t, J=2.2 Hz), 3.85 (4H, t, J=5.0 Hz), 3.25—3.45 (4H, m), 2.69 (s) and 2.55 (t, J=5.0 Hz) 6H, 1.53 (1H, d, J=9.0 Hz), 1.28 (1H, d, J=9.0 Hz)
59	381 (M+), 214, 201, 119, 108	2250, 1710, 1660, 1570, 1540, 1330	8.27 (2H, d, J=4.8 Hz), 6.43 (1H, t, J=4.8 Hz), 4.50 (2H, t, J=1.5 Hz), 3.83 (4H, t, J=4.8 Hz), 3.29 (2H, t, J=1.5 Hz), 2.65—2.45 (8H, m), 1.8—1.35 (8H, m)

TABLE II. (continued)

Compd. No.	Mass spectra m/z	IR spectra cm^{-1} (Nujol)	^{1}H-NMR spectra δ (ppm) ($CDCl_3$)[a,b]
60	399 (M+), 232, 219, 140, 109	2300, 1730, 1680, 1610, 1560, 1490	8.22 (2H, s), 4.53 (2H, t, J=2.2 Hz), 3.46 (4H, t, J=4.8 Hz), 3.32 (2H, t, J=2.2 Hz), 2.6—2.45 (8H, m), 1.80—1.35 (8H, m)
61	355 (M+), 340, 247, 201, 122, 108	2320, 1722, 1680, 1580, 1550	8.33 (2H, d, J=4.8 Hz), 6.47 (1H, t, J=4.8 Hz), 4.53 (2H, t, J=1.8 Hz), 3.85 (4H, t, J=5.3 Hz), 3.30 (2H, t, J=1.8 Hz), 2.55 (t, J=5.3 Hz) and 2.53 (s) 8H, 1.07 (6H, s)
62	409 (M+), 277, 225, 119	2300, 1745, 1675, 1580, 1560, 1440	8.32 (1H, dd, J=2.1, 4.5 Hz), 7.75 (1H, dd, J=2.1, 7.8 Hz), 6.72 (1H, dd, J=4.5, 7.8 Hz), 4.38 (2H, t, J=1.6 Hz), 3.76 (4H, t, J=4.9 Hz), 3.34 (2H, t, J=1.9 Hz), 2.65 (t, J=5.0 Hz) and 2.75—1.7 (m) 12H

a) All compounds were used for the measurement of their NMR spectra as basic form. *b*) Abbreviations: s, singlet; d, doublet; t, triplet; qi, quintet; m, multiplet; dd, double doublet; dt, double triplet. *c*) KBr. *d*) Film.

in quantitative yields by catalytic hydrogenation (method E). The overall yields for the three transformations (V→VII→Ia→Ib) were around 80%. Buspirone was also prepared in 80.5% yield by using this method. This sequence is particularly useful for preparing compounds Ib with a four-carbon chain, since each reaction step proceeds under milder reaction conditions and with less side reactions compared to the routes *via* methods A and B. For instance, preparation of the intermediates (III) in method B always accompanies the formation of 1,4-bisimido-substituted butane as side products. The methodology developed here would also be valuable for the synthesis of buspirone.

Carbinols **32** and **33** were prepared by combinations of three moieties, *i.e.*, the imide (**68**), 3,4-epoxybutyl halide and 1-(2-pyrimidinyl) piperazine (VIa) (Chart 3). Alkylation of VIa with 3,4-epoxybutyl halide, followed by the reaction of the resulting epoxide (**87**) with **68**, afforded **32** (method F), while initial alkylation of **68** with 3,4-epoxybutyl halide and subsequent treatment of the resulting epoxide (**88**) with VIa led to **33** (method G).

As shown in Chart 4, reduction of the imide moiety in **1** with $LiAlH_4$ gave the hydroxylactam derivative (**25**)[9] or the pyrrolidine derivative (**26**),[10] depending on reaction conditions (method H). Conversion of **1** to **25** was achieved under ice-cooling. Treatment of **1** at 40 °C led to the complete reduction product (**26**).

The elemental analysis and spectral data of these newly synthesized compounds (I) are presented in Tables I and II. Starting materials, such as the acid anhydrides (II), the imides (V) and the substituted aryl and heteroaryl piperazine derivatives (VI), if not commercially available, were prepared by known methodologies.[11-13]

Biological Results and Discussion

Although buspirone and related compounds have been shown to be active in conditioned avoidance response (CAR) test in rats, CAR inhibition is evidence of general tranquilizing properties rather than anxiolytic activity.[14] Therefore, our new compounds were evaluated by a procedure highly predictive of anxiolytic activity, a modification[15] of the Vogel[16] or the Geller–Seifter[17] conflict model, in which rats demonstrated attenuation of shock-induced suppression in feeding or drinking behavior. The data are summarized in Table III.

From the initial screening, compound **1** (tandospirone) emerged as an interesting lead. It has been previously reported that the activity of the non-benzodiazepine anxiolytic agent buspirone and ipsapirone is attributed to their selective stimulation of the serotonin-1A (5-HT_{1A}) receptor subtype.[18] Shimizu and coworkers at our research laboratories have demonstrated that **1** binds selectively and with high affinity to 5-HT_{1A} receptors.[19] Therefore, we also employed the receptor-binding methodology to investigate a number of analogues newly synthesized after selection of **1** for further evaluation. The relative affinities of compounds for 5-HT_{1A} receptor binding sites were evaluated on the basis of their ability to displace the 5-HT_{1A} agonist [^{3}H]-8-OH-DPAT [8-hydroxy-2-(di-*n*-propylamino) tetralin].[19] The data are summarized in Table III.

The results obtained in the receptor-binding methodology generally paralleled those obtained in the conflict test in rats. The structure–activity relationships of this series seem to retain several key features observed in a series of buspirone analogues,[5] although several new points emerge from the present study. As expected, considerable variation in the imide structure was permitted so long as the lipophilicity of the imide moiety was retained (compare **1**, **2**, **3**, **5** and **6** with **16** and **23**). Furthermore, the cyclic imide portion of **1** seemed not to be essential for affinity for 5-HT_{1A} sites: compounds **25** and **26** which lack imide structure also displayed the *in vitro* affinities comparable to that of **1**, although their *in vivo* anticonflict activities in rats were less potent than **1**.

The effect of the alkylene chain portion of the molecule between the imide moiety and the arylpiperazine portion was examined with **1** as a standard. The alkyl chain length varied from three to five carbon atoms. In accordance with reported observations,[5] maximum potency was observed with the compound with a four-carbon chain (compare **1** with **27** and **28**). Replacement of the saturated butylene group with a 2-butynylene group caused a loss of activity (**31**). Interestingly, introduction of a double bond with *trans* configuration into the four-carbon chain moiety retained the activity comparable to that of **1**, while the activity decreased if the double bond was situated in *cis* configuration (**29** and **30**). This result may suggest the biologically active conformation of these compounds. Addition of a hydroxy group to the four-carbon chain sharply reduced *in vitro* activity (**32** and **33**). Replacement of the butylene group with a 4-oxobutylene group also resulted in a loss of both *in vivo* and *in vitro* activities (**34**).

The structural modifications of the piperazine ring caused a substantial loss in anticonflict activity. The 5-HT_{1A} receptor affinities of the homopiperazine (**35**) and the 2-methylpiperazine (**36**) were drastically decreased compared to those of 3-methyl and 2,5-dimethylpiperazines (**37**

TABLE III. Pharmacological Activities of Cyclic Imide Derivatives

Compd. No.	Vogel i.p. MED (mg/kg)	Vogel i.p. 2 mg/kg	Vogel i.p. 4 mg/kg	Geller i.p. 2 mg/kg	Geller i.p. 3 mg/kg	5-HT1A binding Ki (nM)
1	5	−	+	+	NT	25.0
2	2.5	−	+	NT	−	18.1
3	1.25	+	−	−	+	4.52
4	>40	−	−	NT	−	268
5	5	+	+	−	+	5.71
6	1.25	+	+	NT	+	7.93
7	10	+	−	NT	−	22.1
8	NT	NT		NT		5.94
9	NT	NT		NT		21.9
10	NT	NT		NT		14.5
11	NT	NT		NT		66.2
12	NT	NT		NT		6.97
13	NT	NT		NT		17.1
14	NT	NT		NT		214
15	NT	−	−	NT	+	128
16	10	+	−	NT	−	68.6
17	<5	−	−	+	NT	3.52
18	2.5	+	+	NT	+	4.12
19	<5	+	−	NT	+	2.58
20	10	+	−	+	NT	26.5
21	10	−	+	−	+	197
22	NT	−	+	NT	−	34.9
23	NT	−	−	NT	−	122
24	NT	−	+	−	−	72.4
25	20	NT		NT		16.3
26	>80	NT		NT		23.9
27	>40	+	+	NT	−	599
28	10	NT		NT		75.5
29	5	NT		NT		8.29
30	40	NT		NT		273
31	>40	NT		NT		823
32	NT	NT		NT		365
33	NT	NT		NT		906
34	>40	NT		NT		>1000
35	>40	NT		NT		1240
36	NT	NT		NT		529
37	>10	NT		NT		85.1
38	>10	NT		NT		67.5
39	10	NT		NT		81.5
40	NT	NT		NT		408
41	5	NT		NT		5.15
42	NT	NT		NT		11.5
43	40	NT		NT		930
44	NT	NT		NT		3210
45	NT	NT		NT		59.9
46	1.25	NT		NT		5.46
47	NT	NT		NT		9.63
48	NT	NT		NT		6.0
49	NT	NT		NT		116
50	5	NT		NT		3.9
51	NT	NT		NT		4.16
52	<5	NT		NT		1.36
53	NT	NT		NT		15.5
Buspirone	5	+	+	NT	+	28.0
Gepirone	5	NT		NT		99
Ipsapirone	5	NT		NT		11.0
Diazepam	NT	+	+	+	NT	NT

NT represents not tested.

and **38**). Replacement of a 2-pyrimidinyl moiety with a 2-pyridyl group or a phenyl group retained both *in vitro* and *in vivo* activities (**46** and **50**). Addition of substituents at the 5-position of 2-pyrimidinyl and 2-pyridyl moieties strongly decreased activity (compare **39**, **40**, **43**, **44**, and **49** with **1** and **46**), while substituents at the 3- or 4-position of these and 2- or 3-position of phenyl moieties appeared to have little effect on the activity (compare **41**, **42**, **47**, **48**, **51**, **52** and **53** with **1**, **46** and **50**).

For comparison, tandospirone (**1**) was examined in animal models which had been used to characterize buspirone. Anxiolytic activity was evaluated as above.

TABLE IV. Pharmacology of SM-3997, Buspirone and Diazepam

Pharmacological activity	SM-3997	Buspirone	Diazepam
Anxiolytic activity (MED: mg/kg)[15]	5	5	2.5
Sedative activity (ED_{50}: mg/kg)[15]	212	263	1.8
Anticonvulsive activity (ED_{50}: mg/kg)[15]	300	300	0.14
Muscle Relaxant activity (ED_{50}: mg/kg)[15]	300	125	9.1
Tranquilizing activity (ED_{50}: mg/kg)[15]	305	43.9	NT

NT represents not tested.

Sedative activity was determined by measuring the ability to potentiate hexobarbital-induced anesthesia,[15] anticonvulsive activity by measuring the ability to suppress the pentylenetetrazole-induced convulsion,[15] muscle relaxant activity by the rota-rod test,[15] and dopamine antagonistic activity by measuring the ability to prevent apomorphine-induced climbing behavior.[15] Pharmacological properties of tandospirone are summarized in Table IV in comparison to those of buspirone and diazepam.

The anxiolytic effect of tandospirone was equal to that of buspirone and one-half that of diazepam. Tandospirone and buspirone were both essentially devoid of sedative, anticonvulsant and muscle relaxant activity, while low doses of diazepam caused sedation and muscle relaxation, and antagonized pentylenetetrazole-induced convulsion. Thus, tandospirone shared with buspirone a similarly favorable pharmacological profile. However, as expected, tandospirone was markedly less potent than buspirone in blocking apomorphine-induced climbing behavior in rats, a model predictive of antipsychotic activity, suggesting that tandospirone may be more anxioselective.

Tandospirone is now undergoing clinical study as a selective anxilolytic agent. After selection of tandospirone for development, several compounds (**3**, **5**, **6**, **17**, **18**, **19**, **41**, **46** and **48**) with higher affinity for $5\text{-}HT_{1A}$ sites were obtained. Follow-up study on these compounds is currently in progress in order to characterize their pharmacological properties.

Experimental

Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Spectra were recorded for all compounds and were consistent with assigned structures. Nuclear magnetic resonance (NMR) spectra were recorded on JEOL GX-270 instruments with tetramethylsilane as an internal standard; chemical shifts are given on the δ scale (ppm), coupling constants (J values) are expressed in herz (Hz), and the following abbreviations are used. s = singlet, d = doublet, t = triplet, q = quartet, qi = quintet, m = multiplet, dd = double doublet and br = broad. Mass spectra (MS) were recorded with Hitachi DF/GC/MS M-80 mass spectrometer. Infrared (IR) spectra were recorded with Hitachi 260-10 IR spectrophotometer. Elemental analysis was determined with a Heraeus elemental analyzer. For column chromatography, Merck Kieselgel 60 (70—230 mesh) was used.

Bicyclo[2.2.1]heptane-2,3-di-*exo*-carboxylic Anhydride (63) A mixture of bicyclo[2.2.1]hept-5-ene-2,3-di-*exo*-carboxylic anhydride[11b] (16.4 g, 0.10 mol) and 50% water-containing 10% palladium on charcoal (0.82 g) in tetrahydrofuran (THF) (263 g) was hydrogenated at room temperature for 1.5 h. Precipitate was removed by filtration, and the filtrate was evaporated under reduced pressure to give **63** (15.93 g, 95.9%), mp 74—78 °C. IR (KBr): 2980, 2960, 2895, 1865, 1835, 1790, 1230, 1195 cm^{-1}.

^{1}H-NMR ($CDCl_3$) δ: 1.1—1.8 (6H, m), 2.7—2.8 (4H, m).

7-Oxabicyclo[2.2.1]heptane-2,3-di-*exo*-carboxylic Anhydride (64) Compound **64** was prepared as described for **63** in quantitative yield, mp 107—109 °C.

Bicyclo[2.2.2]octane-2,3-di-*exo*-carboxylic Anhydride (65) Compound **65** was prepared as described for **63** in 97.4% yield, mp 179—182 °C.

Bicyclo[2.2.1]heptane-2-*exo*-carboxylic-2-*endo*-acetic Anhydride (66) Compound **66** was prepared as described for **63** in 55.0% yield, mp 153—154 °C.

Bicyclo[2.2.2]oct-5-ene-2-*exo*-carboxylic-2-*endo*-acetic Anhydride (67) A mixture of 1,3-cyclohexadiene (6.84 g, 85 mmol) and itaconic anhydride (5.70 g, 51 mmol) in benzene (10 ml) was heated under reflux for 8 h. After cooling, the resulting precipitate was collected by filtration and washed with cooled benzene to give **67** (7.48 g, 73.7%), mp 59—62 °C. IR (Nujol): 3050, 1860, 1790, 1470, 1420, 1230 cm^{-1}.

Bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (68) To a stirred solution of **63** (59.3 g, 0.357 mol) in THF (120 ml) was added 7% aqueous ammonia (133 ml) at room temperature. The mixture was slowly heated to 190 °C, kept for 2 h at the same temperature and cooled. The resulting precipitate was collected by filtration and washed with *n*-hexane to give **68** (47.5 g, 80.5%), mp 153—154 °C. IR (Nujol): 3180, 3060, 1770, 1690, 1360, 1305, 1295, 1279 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.15—1.75 (6H, m), 2.67 (4H, s), 9.0—9.6 (1H, br).

Bicyclo[2.2.1]hept-5-ene-2,3-di-*exo*-carboximide (69) Compound **69** was prepared as described for **68** in 89.4% yield, mp 161—163 °C.

8-Azaspiro[4.5]decane-7,9-dione (70) Compound **70** was prepared as described for **68** in 92.0% yield, mp 155—155.5 °C.

1-(3-Aminopropyl)-4-(2-pyrimidinyl)piperazine (71) To a solution of β-chloropropionitrile (10 g, 0.112 mol) and *N*-(2-pyrimidinyl)piperazine (16.7 g, 0.1 mol) in acetone (10 ml) was added 25% aqueous sodium hydroxide solution (6.4 ml) and the resulting solution was stirred at room temperature for 5 h. After the addition of 30% aqueous sodium hydroxide solution, the solution was extracted with ethyl acetate. The extracts were washed with saturated sodium chloride solution and dried over anhydrous magnesium sulfate and concentrated. The residue was chromatographed on silica gel to give 1-(2-cyanoethyl)-4-(2-pyrimidinyl)piperazine (**72**) (14 g, 64.4%) as a viscous oil. IR (neat): 2930, 2850, 2250, 1590, 1550, 1500, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.4—2.8 (8H, m), 3.85 (4H, t, J= 10.5 Hz), 6.45 (1H, t, J=4.5 Hz), 8.26 (2H, d, J=4.5 Hz). A solution of **72** (5.0 g, 23 mmol) in anhydrous ether (50 ml) was added to a stirred suspension of $LiAlH_4$ (1.0 g, 27.6 mmol) in anhydrous ether (120 ml) at −10 °C under nitrogen. After stirring for 2 h at the same temperature, the reaction mixture was decomposed successively with H_2O (1 ml), 15% aqueous KOH solution (1 ml) and H_2O (3 ml). The organic layer was separated, dried and evaporated under reduced pressure. The residue was chromatographed on silica gel to give 1-(3-aminopropyl)-4-(2-pyrimidinyl)-piperazine (**71**) (3.87 g, 76.0%) as an oil. IR (neat): 3600—3000, 1600, 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.64 (2H, qi, J=6.9 Hz), 2.3—2.85 (10H, m), 3.80 (4H, t, J=10.5 Hz), 6.40 (1H, t, J=4.5 Hz), 8.25 (2H, d, J=4.5 Hz).

1-(4-Aminobutyl)-4-(2-pyrimidinyl)piperazine (73) Compound **73** was prepared as described for **71** in 73.1% yield. IR (neat): 3100—3400, 2920, 1580, 1540, 1480, 1355 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.4—1.65 (4H, m), 1.93 (2H, s), 2.25—2.8 (8H, m), 3.83 (4H, t, J=5.1 Hz), 6.46 (1H, t, J=5.4 Hz), 8.28 (2H, d, J=5.4 Hz).

1-(4-Aminobutyl)-4-(2-pyridyl)piperazine (74) Compound **74** was prepared as described for **71** in quantitative yield. IR (neat): 3100—3600, 1600, 1570, 1490, 1450, 1390 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.1—2.1 (6H, m), 2.25—3.0 (8H, m), 3.4—3.9 (4H, m), 6.45—6.75 (2H, m), 7.25—7.6 (1H, m), 8.05—8.3 (1H, m).

***N*-(4-Bromobutyl)bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (75)** A mixture of **68** (24.8 g, 0.15 mol), 1,4-dibromobutane (162 g, 0.75 mol) and anhydrous K_2CO_3 (30.9 g, 0.225 mol) in acetone (250 ml) was refluxed for 7 h, cooled and filtered. The filtrate was concentrated under reduced pressure (10 mmHg) and the residue was chromatographed on silica gel to give **75** (40.6 g, 90.2%) as an oil. IR (neat): 1765, 1700, 1430, 1395, 1360, 1340, 1295, 1260 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.0—2.1 (10H, m), 2.4—2.9 (4H, m), 3.1—3.7 (4H, m).

***N*-(4-Bromobutyl)bicyclo[2.2.1]hept-5-ene-2,3-di-*exo*-carboximide (76)** Compound **76** was prepared as described for **75** in 76.3% yield as an oil. IR (neat): 1760, 1700, 1435, 1395, 1370, 1140 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.15—2.05 (6H, m), 2.66 (2H, s), 3.2—3.35 (2H, m), 3.40 (2H, t, J=6.8 Hz), 3.48 (2H, t, J=6.8 Hz), 6.1—6.3 (2H, m).

***N*-(4-Bromobutyl)cyclohex-4-ene-1,2-di-*exo*-carboximide (77)** Compound **77** was prepared as described for 75 in 83.3% yield as an oil. IR (neat): 3040, 2950, 2850, 1770, 1700, 1440, 1400, 1370 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.2—3.1 (10H, m), 3.42 (2H, t, J=6.6 Hz), 3.49 (2H, t, J=6.6 Hz), 5.8—6.0 (2H, m).

***N*-(4-Bromopentyl)bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (78)** Compound **78** was prepared as described for **75** in quantitative yield as an oil. IR (neat): 1770, 1700, 1440, 1400, 1370, 1350 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.9—2.1 (12H, m), 2.5—2.8 (4H, m), 3.25—3.6 (4H, m).

***N*-(4-Chloro-2-*trans*-butenyl)bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (79)** A mixture of **68** (3.30 g, 20 mmol), 1,4-dichloro-2-*trans*-butene (15.0 g, 0.12 mol) and anhydrous K_2CO_3 (3.40 g, 24.8 mol) in dimethylformamide (DMF) (35 ml) was heated for 2 h at 90—100 °C. The reaction mixture was filtered and the filtrate was concentrated. The residue was dissolved with toluene (100 ml), washed with H_2O, and dried. The solvent was evaporated and the residue was chromatographed on silica gel to give **79** (4.50 g, 88.7%) as an oil. IR (neat): 2940, 1755, 1690, 1385, 1325, 1165 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.0—1.85 (6H, m), 2.63 (s) and 2.72 (d, J=1.5 Hz) 4H, 4.05 (4H, t, J=5.4 Hz), 5.7—5.95 (2H, m).

***N*-(4-Chloro-2-*cis*-butenyl)bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (80)** Compound **80** was prepared as described for **79** in 90.0% yield. IR (neat): 2940, 1755, 1690, 1385, 1325, 1160 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.0—1.8 (6H, m), 2.60 (s) and 2.70 (d, J=1.5 Hz) 4H, 4.14 (d, J=10.5 Hz) and 4.22 (d, J=4.5 Hz) 4H, 5.7 (2H, m).

***N*-[4-(3-Methyl-1-piperazinyl)butyl]bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (81)** A mixture of **75** (2.0 g, 6.66 mmol) and 2-methylpiperazine (3.3 g, 33.3 mmol) in ethanol (33 ml) was stirred overnight at room temperature. The mixture was concentrated under reduced pressure, and the residue was chromatographed on silica gel to give **81** (2.1 g, 100%) as a yellow oil. IR (neat): 3455, 3300. 1770, 1705, 1445, 1405, 1375, 1330 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.9—1.8 (14H, m), 1.97 (2H, s), 2.15—2.45 (2H, m), 2.45—2.8 (6H, m), 2.8—3.05 (4H, m), 3.35—3.55 (2H, m).

***N*-Propargylbicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (82)** A mixture of **68** (3.30 g, 20.0 mmol), propargyl bromide (2.62 g, 22.0 mmol) and anhydrous K_2CO_3 (3.32 g, 24 mmol) in anhydrous acetone (30 ml) was refluxed with stirring under nitrogen for 1 h, cooled and filtered. The filtrate was concentrated and the residue was recrystallized with *n*-hexane to give **82** (3.70 g, 91%), mp 94—94.5 °C. IR (neat): 3270, 1760, 1685, 1390, 1325, 1180 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.0—1.8 (6H, m), 2.16 (1H, t, J=3.0 Hz), 2.68 (2H, s), 2.76 (2H, d, J=2.0 Hz), 4.13 (2H, d, J=3.0 Hz).

***N*-Propargylbicyclo[2.2.1]hept-5-ene-2,3-di-*exo*-carboximide (83)** Compound **83** was prepared as described for **82** in 93.0% yield, mp 124—126 °C.

8-Propargyl-8-azaspiro[4.5]decane-7,9-dione (84) Compound **84** was prepared as described for **82** in quantitative yield. IR (neat): 3250, 2935, 1720, 1670, 1405, 1370, 1330, 1210, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.4—1.85 (8H, m), 2.12 (1H, t, J=2.4 Hz), 2.63 (4H, s), 4.50 (2H, d, J=2.4 Hz).

***N*-Propargyl-3,3-dimethylglutarimide (85)** Compound **85** was prepared as desribed for **82** in 99.0% yield. IR (neat): 3250, 2950, 1720, 1670, 1410, 1370, 1330, 1220, 1135 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (6H, s), 2.13 (1H, t, J=2.5 Hz), 2.53 (4H, s), 4.50 (2H, d, J=2.5 Hz).

3-Propargylthiazolidine-5-spirocyclopentane-2,4-dione (86) Compound **86** was prepared as described for **82** in 88.8% yield. IR (neat): 3250, 2940, 2110, 1735, 1665, 1405, 1370, 1320, 1145 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.65—2.65 (9H, s), 4.36 (2H, d, J=3.0 Hz).

4-(3,4-Epoxybutyl)-1-(2-pyrimidinyl)piperazine (87) A mixture of 1-(2-pyrimidinyl)piperazine (338 mg, 2.06 mmol), 3,4-epoxybutyl chloride (282 mg, 2.6 mmol) and anhydrous K_2CO_3 (415 mg, 3.0 mmol) in anhydrous acetone (5 ml) was refluxed with stirring under nitrogen for 2 h, cooled and filtered. The filtrate was concentrated and the residue was chromatographed on silica gel to give **87** (272 mg, 60.7%) as an oil. IR (neat): 1590, 1545, 1500, 1450, 1360, 1260, 980 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.6—1.9 (2H, m), 2.4—2.65 (7H, m), 2.78 (1H, t, J=4.5 Hz), 2.85—3.15 (1H, m), 3.83 (4H, t, J=5.0 Hz), 6.43 (1H, t, J=4.8 Hz), 8.29 (2H, d, J=4.8 Hz).

***N*-(3,4-Epoxybutyl)bicyclo[2.2.1]heptane-2,3-di-*exo*-carboximide (88)** A mixture of **68** (330 mg, 2.0 mmol), 3.4-epoxybutyl chloride (282 mg, 2.6 mmol) and anhydrous K_2CO_3 (415 mg, 3.0 mmol) in anhydrous acetone (5 ml) was refluxed with stirring under nitrogen for 2 h, cooled and filtered. The filtrate was concentrated and the residue was chromatographed on silica gel to give **88** (406 mg, 90.1%) as an oil. IR (neat): 2970, 1765, 1710, 1580, 1400, 1365, 1190 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.15—2.05 (8H, m), 2.42 (1H, dd, J=3.0, 5.4 Hz), 2.55—2.75 (5H, m), 2.8—3.0 (1H, m), 3.45—3.8 (2H, m).

2-Methylsulfonyl-4,6-dimethylpyrimidine (89) To a mixture of 2-mercapto-4,6-dimethylpyrimidine (9.8 g, 70 mmol) and sodium hydroxide (3.1 g, 77 mmol) in H_2O (100 ml) was added methyl iodide (10.93 g,

77 mmol) at room temperature. Stirring was continued for 7 h at room temperature. The solution was extracted with dichloromethane. The organic layer was dried and evaporated to give 2-methylthio-4,6-dimethylpyrimidine (90) (9.68 g, 89.7%) as an oil. IR (neat): 1570, 1530, 1430, 1255, 880 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.39 (6H, s), 2.55 (3H, s), 6.62 (1H, s). To a mixture of **90** (4.63 g, 30 mmol) in formic acid (100 ml) was added dropwise 30% aqueous hydrogen peroxide (17 g, 150 mmol) at room temperature. Stirring was continued for 3 h at room temperature. The solution was diluted with H_2O (150 ml) and extracted with dichloromethane. The combined organic layers were dried and concentrated. The residue was recrystallized with isopropyl ether to give 2-methylsulfonyl-4,6-dimethylpyrimidine (**89**) (1.95 g, 34.9%), mp 80—82 °C. IR (Nujol): 1585, 1510, 1300, 1135 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.63 (6H, s), 3.35 (3H, s), 7.28 (1H, s).

2-Methylsulfonyl-4,6-dimethoxypyrimidine (91) Compound **91** was prepared from 2-methylthio-4,6-dihydroxypyrimidine as described for **89** in 89.4% yield. IR (Nujol): 1590, 1522, 1302, 1288, 1270, 1190, 1130 cm^{-1}.

2-Methylsulfonyl-4-methylpyrimidine (92) Compound **92** was prepared from 2-mercapto-4-methylpyrimidine as described for **89** in 36.8% yield. IR (neat): 3000, 2910, 1575, 1530, 1420, 1345, 1310, 1130 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.68 (3H, s), 3.32 (3H, s), 7.43 (1H, d, J=4.8 Hz), 8.62 (1H, d, J=4.8 Hz).

2-Chloro-5-benzyloxypyrimidine (93) A solution of 2-chloro-5-hydroxypyrimidine (310 mg, 2.38 mmol), benzyl bromide (810 mg, 4.75 mmol) and anhydrous K_2CO_3 (394 mg, 2.85 mmol) in anhydrous DMF (20 ml) was heated at 90—100 °C for 1 h. The solution was poured into H_2O and extracted with ethyl acetate. The combined organic layers were washed with brine, dried and evaporated. The residue was chromatographed on silica gel to give **93** (470 mg, 89.7%), mp 76—80 °C. ^{1}H-NMR ($CDCl_3$) δ: 5.10 (2H, s), 7.35 (5H, s), 8.30 (2H, s).

1-(5-Fluoro-2-pyrimidinyl)piperazine (94) To 5-fluorouracil (20.0 g, 0.154 mol) was added phosphorus oxychloride (100 ml, 1.07 mol) and *N*,*N*-diethylaniline (24 ml, 0.225 mol) with stirring at room temperature. After being heated under reflux for 100 min, the mixture was concentrated under reduced pressure. The residue was poured into ice H_2O (300 ml) and extracted with ether. The organic layer were dried and evaporated at 0 °C under reduced pressure to give 2,4-dichloro-5-fluoropyrimidine (**95**) (23.1 g, 89.9%), mp 37—38 °C. ^{1}H-NMR ($CDCl_3$) δ: 8.50 (s). To a solution of **95** (22.0 g, 0.131 mol) in benzene (100 ml) was added activated zinc powder (66 g, 1.01 mol) and 5% ammonium hydroxide in brine (110 ml) with stirring at room temperature. The mixture was heated under reflux with stirring for 6.5 h. After cooling, the mixture was filtered and the filtrate was extracted with benzene. The organic layers were dried and a solution of anhydrous piperazine (57 g, 0.66 mol) in benzene (140 ml) was added to the benzene solution at room temperature. The mixture was stirred under reflux for 4 h, cooled and filtered. The filtrate was concentrated and the residue was distilled to give 1-(5-fluoro-2-pyrimidinyl)piperazine (**94**) (15.97 g, 66.5% from **97**), bp 91—93 °C (0.3 mmHg), mp 38.5—40 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.70 (1H, s), 2.8—3.0 (4H, m), 3.65—3.8 (4H, m), 8.17 (2H, s).

3-Methyl-1-(2-pyrimidinyl)piperazine (96) A mixture of 2-methylpiperazine (5.0 g, 5.0 mmol) and 2-chloropyrimidine (1.14 g, 1.0 mmol) in ethanol (50 ml) was stirred in an ice bath for 30 min and at room temperature for 6 h. The mixture was concentrated under reduced pressure and extracted with AcOEt. The extracts were washed with water and brine, dried and concentrated to give **96** (0.74 g, 41.6%) as an oil. IR (neat): 3280, 1585, 1545, 1495, 1445, 1390, 1355, 1305 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (3H, d, J=11.5 Hz), 1.77 (1H, s), 2.3—3.1 (5H, m), 4.5—4.7 (2H, m), 6.38 (1H, t, J=5.3 Hz), 8.26 (2H, d, J=5.3 Hz).

1-(2-Pyrimidinyl)homopiperazine (97) Compound **97** was prepared as described for **96** in 41.6% yield as an oil. IR (neat): 3300, 1580, 1540, 1500, 1420, 1380, 1360 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.67 (1H, s), 1.7—2.0 (2H, m), 2.7—3.1 (4H, m), 3.7—4.0 (4H, m), 6.38 (1H, t, J=4.8 Hz), 8.28 (2H, d, J=4.8 Hz).

2,5-Dimethyl-1-(2-pyrimidinyl)piperazine (98) Compound **98** was prepared as described for **96** in 11.8% yield as an oil. IR (neat): 2970, 1585, 1545, 1490, 1360, 1000 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05—1.35 (6H, t, J=6.0 Hz), 1.97 (1H, s), 2.63 (1H, dd, J=3.0, 13.9 Hz), 3.15—3.45 (3H, m), 4.25 (1H, dd, J=3.0, 4.5 Hz), 4.65—4.9 (1H, m), 6.42 (1H, t, J=4.8 Hz), 8.30 (2H, d, J=4.8 Hz).

1-(4-Methyl-2-pyrimidinyl)piperazine (99) Compound **99** was prepared as described for **96** in 79.0% yield as an oil. IR (neat): 3180, 1560, 1335, 1252 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.90 (1H, s), 2.34 (3H, s), 2.9 (4H, m), 3.8 (4H, m), 6.35 (1H, d, J=4.0 Hz), 8.17 (1H, d, J=4.0 Hz).

1-(5-Benzyloxy-2-pyrimidinyl)piperazine (100) Compound **100** was prepared as described for **96** in 50.6% yield, mp 95—96 °C.

1-(3-Cyano-2-pyridyl)piperazine (101) Compound **101** was prepared as described for **96** in 96.1% yield, mp 104—106 °C.

1-(3-Chloro-2-pyridyl)piperazine (102) Compound **102** was prepared as described for **96** in 90.8% yield as an oil. IR (neat): 3280, 1575, 1230, 1120, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (1H, s), 2.85—3.1 (4H, m), 3.3—3.5 (4H, m), 6.79 (1H, dd, J=8.0, 4.5 Hz), 7.58 (1H, dd, J=8.0, 3.0 Hz), 8.18 (1H, dd, J=4.5, 3.0 Hz).

1-(5-Chloro-2-pyridyl)piperazine (103) Compound **103** was prepared as described for **96** in 65.7% yield, mp 59—61 °C.

1-(4,6-Dimethyl-2-pyrimidinyl)piperazine (104) Compound **104** was prepared as described for **96** in 65.0% yield, mp 85—86 °C.

1-(4,6-Dimethoxy-2-pyrimidinyl)piperazine (105) Compound **105** was prepared as described for **96** in 48.6% yield, mp 100—103 °C.

1-(5-Bromo-2-pyrimidinyl)piperazine (106) To a mixture of 1-(2-pyrimidinyl)piperazine (3.28 g, 20 mmol) and $CaCO_3$ (1.00 g, 10 mmol) in H_2O (30 ml) was added dropwise bromine (3.52 g, 22 mmol) at 50—55 °C. After being stirred at the same temperature for 2 h, the pH of the resulting solution was ajusted to 10 with 30% aqueous NaOH solution and filtered. The filtrate was extracted with $CHCl_3$ and the organic layers were dried and concentrated. The residue was chromatographed on silica gel to give **106** (3.90 g, 80.2%), mp 64—67 °C. IR (Nujol): 3200, 1570, 1255, 1122, 930 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.9 (4H, m), 3.8 (4H, m), 4.23 (1H, s), 8.35 (2H, s).

(1*R,2*R**,3*S**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (3)** Method A: From **73**. A mixture of bicyclo[2.2.1]heptane-2,3-di-*endo*-carboxylic anhydride (500 mg, 3.00 mmol), **73** (708 mg, 3.00 mmol) and pyridine (12.1 ml) was refluxed for 5 h. The mixture was concentrated under reduced pressure and the residue was chromatographed on silica gel to give **3** as an oil. Treatment of the free base **3** with 6% hydrogen chloride–isopropyl alcohol (IPA) and recrystallization from IPA gave the hydrochloride of **3** (920 mg, 67.2%), mp 202—203 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (1)** Method B: From **75**. To a mixture of *N*-(2-pyrimidinyl)piperazine (34.8 g, 212 mmol), anhydrous K_2CO_3 (32.2 g, 233 mmol) and potassium iodide (3.87 g, 23.3 mmol) in anhydrous DMF (700 ml) was added **75** (70.0 g, 233 mmol) with stirring and the mixture was stirred at 90 °C for 1 h. After being cooled, the mixture was poured into H_2O (2100 ml) and extracted with four 500 ml portions of EtOAc. The organic layers were washed with H_2O (300 ml) and brine (300 ml), dried and concentrated. Recrystallization of the residue from a mixture of toluene and *n*-hexane gave **1** (70.1 g, 81.3%), mp 112—113.5 °C. Treatment of the free base **1** with citric acid gave the citrate, mp 169.5—170 °C

Method E: From **31**. A solution of **31** (380 mg, 1.0 mmol) in THF (10 ml) was hydrogenated over 10% palladium on charcoal (25 mg) at room temperature for 1.5 h. After removal of the catalyst, the filtrate was concentrated under reduced pressure and chromatographed to give **1**. Treatment of the free base **1** with citric acid gave its citrate (0.51 g, 88.6%), mp 169.5—170 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[3-Methyl-4-(2-pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (36)** Method C: A mixture of **81** (1.00 g, 3.13 mmol), 2-chloropyrimidine (0.72 g, 6.26 mmol) and anhydrous K_2CO_3 (0.87 g, 6.26 mmol) in anhydrous dimethylsulfoxide (DMSO) (10 ml) was heated at 90—100 °C for 3.5 h. After being cooled, the mixture was poured into H_2O (50 ml) and extracted with ethyl acetate (200 ml). The organic extracts were washed successively with water and brine, dried and concentrated. The residue was chromatographed on silica gel to give **36** as an oil. Treatment of the free base **36** with 6% hydrogen chloride–IPA and recrystallization from IPA gave the hydrochloride of **36** (560 mg, 44.8%), mp 116—117 °C (dec.).

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]-2-*trans*-butenyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (29)** To a stirred mixture of 1-(2-pyrimidinyl)piperazine (0.65 g, 4.0 mmol) and anhydrous K_2CO_3 (0.80 g, 5.8 mmol) in anhydrous DMF (15 ml) was added **79** (1.02 g, 4.0 mmol) and the mixture was heated at 90—100 °C for 2 h. After evaporation of the solvent, the mixture was extracted with toluene and the organic extracts were washed with water (300 ml), dried and concentrated. The residue was chromatographed on silica gel to give **29** (1.33 g, 86.8%) as an oil, chromatographed on silica gel to give **29** (1.33 g, 86.8%) as an oil, mp of its citrate 150—152 °C (dec.).

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]-2-butynyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (31)** Method D: To a stirred solution of **82** (0.41 g, 2.00 mmol), 1-(2-pyrimidinyl)piperazine (331 mg,

2.01 mmol) and a 35% aqueous formaldehyde solution (0.33 ml) in dioxane (2 ml) was added dropwise a solution of copper sulfate (18 mg, 0.11 mmol) in H_2O (1 ml) at room temperature. The mixture was heated with stirring at 70—80 °C for 70 min. After evaporation of the solvents, the residue was diluted with toluene. The insoluble materials were removed by filtration, and the toluene was evaporated. The residue was chromatographed on silical gal to give **31** (0.754 g, 99.4%), mp 122—123 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Methoxyphenyl)-1-piperazinyl]butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (52)** Compound **52** was prepared from **57** as described for the preparation of **1** from **31** (method E) in 90.5% yield as a crystal, mp 92.5—93.5 °C.

***N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]bicyclo[2.2.2]octane-2-*exo*-carboxy-2-*endo*-acetimide (18)** Compound **18** was prepared from *N*-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]bicyclo[2.2.2]oct-5-ene-2-*exo*-carboxy-2-*endo*-acetimide **(19)** as described for the preparation of **1** from **31** (method E) in 82.6% yield, mp of its hydrochloride 245—247 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(5-Hydroxy-2-pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (43)** A solution of (1*R**,2*S**,3*R**,4*S**)-*N*-[4-[4-(5-Benzyloxy-2-pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide **(44)** (110 mg, 0.22 mmol) in MeOH (30 ml) was hydrogenated over 10% palladium on charcoal (6 mg) at 8 atm of hydrogen and 100 °C for 2 h. The catalyst was removed by filtration, and the filtrate was evaporated under reduced pressure. The residue was chromatographed to give **43** (70 mg, 78.0%), mp 198—200 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[2-Hydroxy-4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (32)** Method F: A mixture of **87** (1.587 g, 7.08 mmol), **68** (2.339 g, 14.16 mmol) and powdered anhydrous K_2CO_3 (2.935 g, 21.24 mmol) in *n*-butanol (40 ml) was refluxed for 6 h. The reaction mixture was concentrated under reduced pressure and the residue was recrystallized from IPA to give **32** (2.351 g, 59.0%), mp 122—124.5 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[3-Hydroxy-4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (33)** Method G: A mixture of **88** (1.968 g, 8.74 mmol) and 1-(2-pyrimidinyl)piperazine (1.58 g, 9.62 mmol) in *n*-butanol (25 ml) was refluxed for 4 h. The reaction mixture was concentrated under reduced pressure and the residue was recrystallized from $CHCl_3$–Et_2O to give **33** (2.798 g, 82.2%), mp 119—120 °C.

(1*R,2*S**,3*R**,4*S**)-4-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]-4-azatricyclo[5.2.1.$0^{2,6}$]decane (26)** To a stirred mixture of $LiALH_4$ (128 mg, 3.38 mmol) in anhydrous THF (5 ml) was added a solution of **1** (0.50 g, 1.13 mmol) in anhydrous THF (5 ml) at room temperature. The mixture was stirred at room temperature for 2.5 h and cooled in an ice bath. After the careful decomposition of excess $LiALH_4$ with cold H_2O, the resulting mixture was extracted with AcOEt. The organic extracts were washed with H_2O and brine, dried and concentrated. The residue was treated with 12% hydrogen chloride–IPA to give the hydrochloride of **26** (420 mg, 68.9%), mp 294—296 °C.

(1*R,2*S**,3*R**,4*S**)-5-Hydroxy-4-[4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl]-4-azatricyclo[5.2.1.$0^{2,6}$]decan-3-one (25)** To a mixture of **1** (3.00 g, 7.83 mmol), IPA (48 ml) and H_2O (12 ml) was added a solution of $NaBH_4$ (0.71 mg, 18.8 mmol) in 0.1% aqueous NaOH (4.8 ml) at room temperature. The mixture was refluxed for 2 h and cooled in an ice bath. Cold H_2O was added into the reaction mixture and the resulting mixture was extracted with $CHCl_3$. The organic extracts were washed with H_2O and brine, dried, concentrated and crystallized from IPA to give **25** (2.20 g, 72.9%), mp 144—147 °C.

3-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]-2,4-(1*H*,3*H*)-quinazolinedione (22) To a solution of **73** (1.00 g, 4.25 mmol) in anhydrous DMF (5 ml) was added isatoic anhydride (1.13 g, 4.78 mmol) at room temperature. The mixture was stirred over night at room temperature. After the addition of 30% aqueous NaOH the solution was extracted with $CHCl_3$. The organic extracts were washed with brine, dried and concentrated. The residue was chromatographed to give 1-(4-anthraniloylaminobutyl)-4-(2-pyrimidinyl)piperazine **(107)** (0.23 g, 15.3%) as an oil. IR (neat): 3440, 3325, 2945, 2850, 2810, 1630, 1590, 1510 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.4—1.8 (4H, m), 2.2—3.7 (6H, m), 3.15—3.65 (2H, m), 3.65—4.95 (4H, m), 5.1—5.9 (1H, m), 6.2—6.9 (3H, m), 7.3 (4H, s), 8.25 (2H, d, J = 5.3 Hz). To a solution of **107** (230 mg, 0.65 mmol) in anhydrous THF (3 ml) was added dropwise ethyl chloroformate (74 mg, 0.68 mmol) with stirring at room temperature. The mixture was heated in an oil bath at 200—235 °C for 5 h and cooled. After addition of 30% aqueous NaOH, the mixture was extracted with $CHCl_3$. The organic extracts were washed with brine, dried and concentrated. The residue was chromatographed to give 3-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,4-(1*H*,3*H*)quinazolinedione **(22)** (0.10 g, 40.5%), mp of its hydrochloride 280—283 °C.

(1*R,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]-4-oxobutyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (34)** A mixture of **63** (3.32 g, 20.0 mmol) and γ-aminobutyric acid (2.06 g, 20.0 mmol) was heated at 150 °C for 30 min. The mixture was cooled and recrystallized from toluene–isopropyl ether to give (1*R**,2*S**,3*R**,4*S**)-*N*-(3-carboxypropyl)-2,3-bicyclo[2.2.1]heptanedicarboximide **(108)** (3.50 g, 69.6%), mp 79—81 °C. IR (Nujol): 3070, 2300—2700, 1735, 1670, 1410 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.09 (1H, d, J = 11.0 Hz), 1.21 (1H, d, J = 11.0 Hz), 1.32 (2H, d, J = 6.0 Hz), 1.66 (2H, d, J = 6.0 Hz), 1.87 (2H, q, J = 7.5 Hz), 2.37 (2H, t, J = 7.5 Hz), 2.60 (2H, s), 2.70 (2H, s), 3.53 (2H, t, J = 7.5 Hz), 10.23 (1H, s).

A mixture of **108** (2.00 g, 7.97 mmol) and thionyl chloride (20 ml) was heated under reflux for 4 h. The excess thionyl chloride was evaporated and the residue was dissolved in anhydrous THF (21 ml). The solution was added dropwise to a solution of 1-(2-pyrimidinyl)piperazine (1.40 g, 8.36 mmol) and triethylamine (1.70 g, 16.7 mmol) in anhydrous THF (14 ml). After being stirred for 10.5 h, the mixture was filtered and the filtrate was concentrated. The residue was chromatographed to give (1*R**,2*S**,3*R**,4*S**)-*N*-[4-[4-(2-pyrimidinyl)-1-piperazinyl]-4-oxobutyl]-2,3-bicyclo[2.2.1]heptanedicarboximide (34) as an oil. Treatment of the oil with 12% hydrogen chloride–IPA and recrystallization gave the hydrochloride of **34** (2.00 g, 54.1%), mp 185—188 °C.

8-[4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro-[4.5]decane-7.9-dione (buspirone) A mixture of 8-azaspiro[4.5]decane-7,9-dione (1.67 g, 10.0 mmol), propargyl bromide (2.62 g, 22.0 mmol) and anhydrous K_2CO_3 (3.32 g, 24 mmol) in anhydrous acetone (25 ml) was refluxed with stirring under nitrogen for 10 h, cooled and filtered. The filtrate was concentrated to give **84** in quantitative yield. IR (neat): 3250, 2935, 1720, 1670, 1405, 1370, 1330, 1210, 1130 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.4—1.85 (8H, m), 2.12 (1H, t, J = 2.4 Hz), 2.63 (4H, s), 4.50 (2H, d, J = 2.4 Hz).

To a stirred solution of **84** (0.39 g, 1.70 mmol), 1-(2-pyrimidinyl)piperazine (334 mg, 2.04 mmol) and a 35% aqueous formaldehyde solution (0.35 ml) in dioxane (4 ml) was added dropwise a solution of cupric chloride dihydrate (20 mg, 0.12 mmol) in H_2O (2 ml) at room temperature. The mixture was heated with stirring at 70—80 °C for 1.5 h. After evaporation of the solvents, the residue was diluted with toluene. The insoluble materials were removed by filtration, and the filtrate was washed with brine and extracted with dil. hydrochloric acid. The acidic extracts were neutralized with saturated $NaHCO_3$ solution and extracted with $CHCl_3$. The $CHCl_3$ extracts evaporated to give curde 8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]-2-butynyl]-8-azaspiro[4.5]decane-7,9-dione **(59)** (698 mg). A solution of crude **59** (698 mg) in THF (30 ml) was hydrogenated over 10% palladium on charcoal (90 mg) at room temperature for 50 min. After removal of the catalyst, the filtrate was concentrated under reduced pressure and chromatographed to give free base of buspirone. Treatment of the free base with hydrogen chloride in IPA gave buspirone (522 mg, 80.5% from 8-azaspiro[4.5]decane-7,9-dione), mp 200—202 °C (mp of standard sample 201—203 °C).

Anxiolitic Activity Anticonflict Activity: *In vivo* anxiolitic activity of the target compounds was determined by the modified method of Vogel anticonflict method[17] and Geller anticonflict method,[16] as previously reported.[15]

Serotonin-1A (5-HT_{1A}) Binding Activity: *In vitro* anxiolytic activity of the target compounds was determined by measuring the ability to displace serotonin-1A (5-HT_{1A}) receptor radioligand, as previously reported.[19]

Sedative Activity: Sedative activity was determined by measuring the ability to potentiate the hexobarbital-induced anesthesia, as previously reported.[15]

Anticonvulsive Activity: Anticonvulsive activity was determined by measuring the ability to suppress the pentylenetetrazole-induced convulsion, as previosly reported.[15]

Muscle Relaxant Activity: Muscle relaxant activity was determined by the rota-rod test, as previously reported.[15]

Tranquilizing Activity: *In vivo* tranquilizing activity was determined by measuring the ability to prevent apomorphine-induced climbing behavior, as previously reported.[15]

Acknowledgements The authors are grateful to Drs. M. Nakamura, A. Hirose and H. Shimizu for biological assays and to Mr. I. Maruyama and Miss M. Yoshigi for synthetic collaboration.

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Synthesis and Antifungal Activities of a Series of (1,2-Disubstituted Vinyl)imidazoles

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A series of vinylimidazoles containing a hetero atom such as sulfur or oxygen at a β-position of the vinyl group was prepared and the antifungal activities were tested. It was found that sulfur-substituted derivatives such as (*E*)-1-[2-(methylthio)-1-[2-(pentyloxy)phenyl]ethenyl]-1*H*-imidazole (5a-5) and (*E*)-1-[1-[2-(hexyloxy)phenyl]-2-(methylthio)ethenyl]-1*H*-imidazole (5a-6) showed excellent antifungal activities against dermatophytes and yeast cells. The stereochemistry of the hydrochloride salt of 5a-5 was determined by X-ray crystallography. The structure–activity relationships were discussed.

Keywords antifungal activity; imidazole; X-ray crystallography; antifungal agent; structure–activity relationships

Imidazole derivatives have been widely investigated as antifungal agents for plant pathogenic fungi and dermatophytes since the late 1970's.[1] Some imidazole-based antifungal agents are now clinically used, and these are classified into three general groups on the basis of chemical structures: the arylmethylimidazoles, the arylethylimidazoles and the vinylimidazoles.[2] We have synthesized a new series of vinylimidazole derivatives containing a hetero atom such as sulfur or oxygen at a β-position of the vinyl group in order to obtain more potent and broad spectrum antifungal agents, and found that sulfur-substituted *E*-form derivatives were more active than the others against a variety of fungi including dermatopytes and yeast cells. In these compounds, **5a**-5 and **5a**-6 showed the most excellent antifungal activities. The minimum inhibitory concentrations (MICs) of both **5a**-5 and **5a**-6 were *Candida albicans* 12.5 μg/ml, *Trichophyton mentagrophytes* 0.2 μg/ml, *Trichophyton tonsurans* 0.2 μg/ml, *Trichophyton rubrum* 0.2 μg/ml and *Microsporum gypseum* 0.2 μg/ml *in vitro*, respectively. The stereochemistry of the hydrochloride salt of **5a**-5 was determined by X-ray crystallography. In this paper, we wish to report the synthesis, characterization and antifungal activities of these synthetic compounds.

Synthesis The preparations of sulfur-substituted vinylimidazoles were carried out by the following method (Chart 1). Treatment of 2-bromo-2′-hydroxyacetophenone (**1a**) with methylmercaptan in base gave 2′-hydroxy-2-(methylthio)acetophenone (**2a**). The reaction of **2a** with 1,1′-diimidazolyl sulfoxide by the method of Ogata *et al.*[3] gave two geometric isomers, (*E*)-1-[1-(2-hydroxyphenyl)-2-(methylthio)ethenyl]-1*H*-imidazole (**3a**) and its *Z*-isomer (**4a**) in the ratio of about 5:1, which were separated by column chromatography. Ogata *et al.*[4] reported that the chemical shifts of the vinyl protons of *E*- and *Z*-1-[1-(2-hydroxyphenyl)-2-methylvinyl]-1*H*-imidazole were 6.01 and 6.28 ppm, respectively. Because the chemical shifts of the vinyl protons of **3a** and **4a** were 6.77 and 7.08, we presumed the configurations of the two isomers to be *E* and *Z*, respectively. In the same manner as above, **3b**—**f** (*E*-isomers) and **4b**—**f** (*Z*-isomers) were also prepared in the ratio of about 5:1. Equilibrium mixture of **3a** and **4a** was obtained by heating, and their ratio was about 2:3 by

150°C, 3a

1a : R^1=H
1b : R^2=Cl

1) R^2SH
2) imidazole, $SOCl_2$

3a—f   4a—f

R^3X

5a—f   6a, e

a : R^1=H, R^2=Me **b** : R^1=H, R^2=Et **c** : R^1=H, R^2=Pr **d** : R^1=H, R^2=Bu **e** : R^1=H, R^2=Ph **f** : R^1=Cl, R^2=Me

Chart 1

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Chart 2

TABLE I. Structures and Antifungal Activities of 5

No.	R^1	R^2	R^3	Formula	HRMS Calcd (Found)	MIC (μg/ml)[a] *C. albi*	*T. menta*	*T. tons*	*T. rub*	*M. gyp*
5a-1	H	Me	Me	$C_{13}H_{14}N_2OS$	246.0827 (246.0828)	>25	12.5	3.12	1.56	12.5
5a-2	H	Me	Et	$C_{14}H_{16}N_2OS$	260.0983 (260.1014)	>25	1.56	0.78	0.39	3.12
5a-3	H	Me	Pr	$C_{15}H_{18}N_2OS$	274.1139 (274.1158)	>25	0.78	0.39	0.39	1.56
5a-4	H	Me	Bu	$C_{16}H_{20}N_2OS$	288.1296 (288.1306)	>25	0.20	<0.20	<0.20	0.20
5a-5	H	Me	Pen	$C_{17}H_{22}N_2OS$	302.1453 (302.1501)	12.5	<0.20	<0.20	<0.20	<0.20
5a-6	H	Me	Hex	$C_{18}H_{24}N_2OS$	316.1609 (316.1568)	12.5	<0.20	<0.20	<0.20	<0.20
5a-7	H	Me	Heptyl	$C_{19}H_{26}N_2OS$	330.1766 (330.1730)	25	<0.20	<0.20	<0.20	<0.20
5a-8	H	Me	Octyl	$C_{20}H_{28}N_2OS$	344.1922 (344.1941)	>25	0.39	<0.20	<0.20	3.12
5a-9	H	Me	Bn	$C_{19}H_{18}N_2OS$	322.1141 (322.1122)	>25	1.56	0.78	1.56	3.12
5a-10	H	Me	Bn (2,4-diCl)	$C_{19}H_{16}Cl_2N_2OS$	390.0359 (390.0310)	>25	0.20	0.20	<0.20	0.78
5b-1	H	Et	Me	$C_{14}H_{16}N_2OS$	260.0983 (260.0953)	>25	12.5	6.25	6.25	25
5b-2	H	Et	Et	$C_{15}H_{18}N_2OS$	274.1139 (274.1138)	>25	3.12	0.78	0.78	6.25
5b-3	H	Et	Pr	$C_{16}H_{20}N_2OS$	288.1296 (288.1302)	>25	1.56	0.78	0.39	1.56
5b-4	H	Et	Bu	$C_{17}H_{22}N_2OS$	302.1453 (302.1513)	25	0.39	0.20	<0.20	0.78
5b-5	H	Et	Pen	$C_{18}H_{24}N_2OS$	316.1609 (316.1615)	25	<0.20	<0.20	<0.20	<0.20
5b-6	H	Et	Hex	$C_{19}H_{26}N_2OS$	330.1766 (330.1758)	25	0.39	<0.20	<0.20	0.78
5b-7	H	Et	Heptyl	$C_{20}H_{28}N_2OS$	344.1922 (344.1970)	>25	1.56	0.20	0.20	3.12
5c-1	H	Pr	Me	$C_{15}H_{18}N_2OS$	274.1140 (274.1092)	>25	6.25	3.12	3.12	6.25
5c-2	H	Pr	Et	$C_{16}H_{20}N_2OS$	288.1296 (288.1274)	>25	1.56	0.78	0.78	1.56
5c-3	H	Pr	Pr	$C_{17}H_{22}N_2OS$	302.1453 (302.1444)	12.5	0.20	0.20	<0.20	0.39
5c-4	H	Pr	Bu	$C_{18}H_{24}N_2OS$	316.1609 (316.1562)	12.5	<0.20	0.20	<0.20	<0.20
5c-5	H	Pr	Pen	$C_{19}H_{26}N_2OS$	330.1766 (330.1806)	12.5	<0.20	<0.20	0.20	0.20
5d-1	H	Bu	Me	$C_{16}H_{20}N_2OS$	288.1296 (288.1340)	25	3.12	0.78	1.56	6.25
5d-2	H	Bu	Et	$C_{17}H_{22}N_2OS$	302.1453 (302.1431)	25	0.20	<0.20	<0.20	0.20
5d-3	H	Bu	Pr	$C_{18}H_{24}N_2OS$	316.1610 (316.1600)	25	<0.20	<0.20	<0.20	<0.20
5d-4	H	Bu	Bu	$C_{19}H_{26}N_2OS$	330.1766 (330.1773)	25	<0.20	<0.20	<0.20	0.39
5d-5	H	Bu	Pen	$C_{20}H_{28}N_2OS$	344.1922 (344.1904)	>25	1.56	<0.20	0.20	1.56
5e-1	H	Ph	Et	$C_{19}H_{18}N_2OS$	322.1140 (322.1095)	25	0.78	0.78	0.39	3.12
5e-2	H	Ph	Pr	$C_{20}H_{20}N_2OS$	336.1296 (336.1282)	>25	0.78	1.56	<0.20	3.12
5e-3	H	Ph	Bu	$C_{21}H_{22}N_2OS$	350.1453 (350.1471)	>25	3.12	6.25	1.56	6.25
5f-1	Cl	Me	Pr	$C_{15}H_{17}ClN_2OS$	308.0750 (308.0773)	>25	0.78	0.39	0.39	1.56
5f-2	Cl	Me	Bu	$C_{16}H_{19}ClN_2OS$	322.0907 (322.0935)	>25	<0.20	<0.20	<0.20	0.39
5f-3	Cl	Me	Pen	$C_{17}H_{21}ClN_2OS$	336.1064 (336.1016)	>25	0.20	<0.20	<0.20	1.56

a) *C. albi*: *Candida albicans* NHL 4019, *T. menta*: *Trichophyton mentagrophytes* QM 248, *T. tons*: *Trichophyton tonsurans* IFO 5928, *T. rub*: *Trichophyton rubrum* NHL J, *M. gyp*: *Microsporum gypseum* IFO 8231.

high performance liquid chromatography (HPLC). To get both alkylated derivatives of **3a** and **4a**, the minor product **4a** was also prepared from **3a**. Heating of **3a** in diethylene glycol dimethyl ether at reflux temperature, followed by recrystallization, gave **4a** in 34% yield. Treatment of **3a** and **4a** with alkyl halides in the presence of alkali gave **5a**-1-10 and **6a**-1-3 in moderate yields, respectively. The stereochemistry of **5a** was confirmed by X-ray crystallography of its hydrochloride salt (Experimental section). Treatment of **3b**—**e** and **4e** with alkyl halides in the presence of alkali also gave **5b**—**e** (*E*-isomers) and **6e** (*Z*-isomers), respectively. The derivatives **5f**-1-3, which were substituted by the chloro group at the 5 position of the benzene ring, were also prepared.

Oxygen-substituted vinylimidazoles were obtained by the following method (Chart 2). Treatment of 2-(benzyloxy)-phenylmagnesium bromide with methoxyacetonitrile in tetrahydrofuran (THF) gave 2′-benzyloxy-2-methoxyacetophenone (**8**) in 57% yield. Reduction of **8** with Pd catalyst under hydrogen atmosphere gave 2′-hydroxy-2-methoxyacetophenone (**9**) in a quantitative yield. The reaction of **9** with 1,1′-diimidazolyl sulfoxide gave the single product **10** in 72% yield. Treatment of **10** with alkyl halides in the presence of alkali gave its *O*-alkyl derivatives (**11**-1-4).

Results and Discussion

The antifungal activities of the compounds in this study are summarized in Tables I (**5**-series), II (**6**-series), III (**11**-series). In the series of (*E*)-2-methylsulfides (**5a**-1-10), **5a**-5 and **5a**-6 were found to be most active against both dermatophytes and yeast cells. In this series, 2′-*O*-benzyl derivatives (**5a**-9, **5a**-10) were less active than corresponding 2′-*O*-pentyl (**5a**-5) or 2′-*O*-hexyl (**5a**-6) derivatives.

In the series of (*E*)-2-ethylsulfinyl (**5b**-1-7), (*E*)-2-propylsulfinyl (**5c**-1-5) and (*E*)-2-butylsulfinyl (**5d**-1-5) de-

TABLE II. Structures and Antifungal Activities of **6**

No.	R^1	R^2	R^3	Formula	HRMS Calcd (Found)	MIC (μg/ml)[a]				
						C. albi	*T. menta*	*T. tons*	*T. rub*	*M. gyp*
6a-1	H	Me	Bu	$C_{16}H_{20}N_2OS$	288.1297 (288.1347)	25	0.78	0.39	0.39	0.78
6a-2	H	Me	Pen	$C_{17}H_{22}N_2OS$	302.1453 (302.1497)	25	<0.20	<0.20	<0.20	0.39
6a-3	H	Me	Hex	$C_{18}H_{24}N_2OS$	316.1609 (316.1583)	25	<0.20	<0.20	<0.20	0.39
6e-1	H	Ph	Et	$C_{19}H_{18}N_2OS$	322.1139 (322.1170)	>25	6.25	>25	3.12	>25
6e-2	H	Ph	Pr	$C_{20}H_{20}N_2OS$	336.1297 (336.1301)	>25	6.25	6.25	6.12	12.5
6e-3	H	Ph	Bu	$C_{21}H_{22}N_2OS$	350.1453 (350.1457)	>25	>25	>25	>25	>25

a) C. albi: *Candida albicans* NHL 4019, *T. menta*: *Trichophyton mentagrophytes* QM 248, *T. tons*: *Trichophyton tonsurans* IFO 5928, *T. rub*: *Trichophyton rubrum* NHL J, *M. gyp*: *Microsporum gypseum* IFO 8231.

TABLE III. Structures and Antifungal Activities of **11**

No.	R^4	Formula	HRMS Calcd (Found)	MIC (μg/ml)[a]				
				C. albi	*T. menta*	*T. tons*	*T. rub*	*M. gyp*
11-1	Pr	$C_{15}H_{18}N_2O_2$	258.1368 (258.1394)	>25	12.5	12.5	3.12	12.5
11-2	Bu	$C_{16}H_{20}N_2O_2$	272.1525 (272.1488)	>25	6.25	6.25	3.12	6.25
11-3	Pen	$C_{17}H_{22}N_2O_2$	286.1682 (286.1662)	>25	3.12	3.12	0.78	3.12
11-4	Hex	$C_{18}H_{24}N_2O_2$	300.1838	>25	1.56	1.56	0.78	3.12

a) C. albi: *Candida albicans* NHL 4019, *T. menta*: *Trichophyton mentagrophytes* QM 248, *T. tons*: *Trichophyton tonsurans* IFO 5928, *T. rub*: *Trichophyton rubrum* NHL J, *M. gyp*: *Microsporum gypseum* IFO 8231.

TABLE IV. Physical Properties and Spectral Data of **3** and **4**

No.	R^1	R^2	Formula	HRMS Calcd (Found)	mp (°C) (Recry. solv.[a])	IR (cm^{-1}) (KBr)	NMR: δ ppm *J* (Hz) (solv.[b])
3a	H	Me	$C_{12}H_{12}N_2OS$	232.0670 (232.0640)	201—203 (C, M)	1455 1245	(S) 2.30 (3H, s, SMe), 6.77 (1H, s, =CH), 6.85—7.40 (6H, m, ArH + imidaC4, C5-H), 7.74 (1H, br s, imidaC2-H), 9.70 (1H, br, OH)
3b	H	Et	$C_{13}H_{14}N_2OS$	246.0827 (246.0851)	154—156 (M)	1495 1230	(D) 1.30 (3H, t, *J*=7, Me), 2.74 (2H, q, *J*=7, SCH_2), 6.42 (1H, s, =CH), 6.67—7.38 (6H, m, ArH + imidaC4, C5-H), 7.48 (1H, br s, imidaC2-H), 8.80 (1H, br, OH)
3c	H	Pr	$C_{14}H_{16}N_2OS$	260.0983 (260.1026)	127—128 (M)	1495 1230	(D) 0.98 (3H, t, *J*=7, Me), 1.26—1.85 (2H, m, CH_2), 2.68 (2H, t, *J*=7, SCH_2), 6.40 (1H, s, =CH), 6.65—7.40 (6H, m, ArH + imidaC4, C5-H), 7.46 (1H, br s, imidaC2-H), 8.50 (1H, br, OH)
3d	H	Bu	$C_{15}H_{18}N_2OS$	274.1140 (274.1137)	116—118 (EA, H)	1495 1230	(D) 0.88 (3H, t, *J*=7, Me), 1.02—2.00 (4H, m, $CH_2 \times 2$), 2.69 (2H, t, *J*=7, SCH_2), 6.40 (1H, s, =CH), 6.61—7.30 (6H, m, ArH + imidaC4, C5-H), 7.42 (1H, br s, imidaC2-H), 8.38 (1H, br, OH)
3e	H	Ph	$C_{17}H_{14}N_2OS$	294.0827 (294.0843)	152—154 (M)	1450 1230	(A) 6.65 (1H, s, =CH), 6.63—7.40 (11H, m, ArH + imidaC4, C5-H), 7.47 (1H, br s, imidaC2-H)
3f	Cl	Me	$C_{12}H_{11}ClN_2OS$	266.0281 (266.0310)	193—195 (M)	1495 1240	(S) 2.33 (3H, s, S-Me), 6.82 (1H, s, =CH), 6.56—7.12 (5H, m, ArH + imidaC4, C5-H), 7.60 (1H, br s, imidaC2-H), 10.06 (1H, br, OH)
4a	H	Me	$C_{12}H_{12}N_2OS$	232.0670 (232.0623)	206—208 (M)	1450 1250	(S) 2.39 (3H, s, SMe), 7.08 (1H, s, =CH), 6.45—7.24 (6H, m, ArH + imidaC4, C5-H), 7.66 (1H, br s, imidaC2-H), 10.03 (1H, br, OH)
4b	H	Et	$C_{13}H_{14}N_2OS$	246.0827 (246.0851)	195—197 (M)	1495 1230	(D) 1.25 (3H, t, *J*=7, Me), 2.77 (2H, q, *J*=7, SCH_2), 6.80 (1H, s, =CH), 6.75—7.40 (6H, m, ArH + imidaC4, C5-H), 7.68 (1H, br s, imidaC2-H), 9.08 (1H, br, OH)
4c	H	Pr	$C_{14}H_{16}N_2OS$	260.0984 (260.0936)	191—192 (M)	1495 1230	(D) 1.01 (3H, t, *J*=7, Me), 1.20—1.82 (2H, m, CH_2), 2.77 (2H, t, *J*=7, SCH_2), 6.80 (1H, s, =CH), 6.83—7.40 (6H, m, ArH + imidaC4, C5-H), 7.69 (1H, br s, imidaC2-H), 9.65 (1H, br, OH)
4d	H	Bu	$C_{15}H_{18}N_2OS$	274.1140 (274.1093)	166—167 (M)	1495 1230	(D) 0.88 (3H, t, *J*=7, Me), 1.08—2.00 (4H, m, $CH_2 \times 2$), 2.76 (2H, t, *J*=7, SCH_2), 6.82 (1H, s, =CH), 6.86—7.37 (6H, m, ArH + imidaC4, C5-H), 7.65 (1H, br s, imidaC2-H), 8.96 (1H, br, OH)
4e	H	Ph	$C_{17}H_{14}N_2OS$	294.0827 (294.0757)	235—237 (M)	1440 1245	(D) 6.85 (1H, s, =CH), 7.00—7.53 (12H, m, ArH + imidaH), 9.26 (1H, br, OH)
4f	Cl	Me	$C_{12}H_{11}ClN_2OS$	266.0282 (266.0309)	250—252 (M)	1485 1245	(S) 2.41 (3H, s, S-Me), 7.10 (1H, s, =CH), 6.54—7.33 (5H, m, ArH + imidaC4, C5-H), 7.66 (1H, br s, imidaC2-H), 10.35 (1H, br, OH)

a) C = chloroform, M = methanol. *b)* S = d_6-DMSO, D = $CDCl_3$, A = CD_3OD.

TABLE V. Physical Properties and Spectral Data of **5**, **6** and **11**

No.	Yield (%)	mp (°C) (Recry. solv.[a])	IR (cm^{-1}) (KBr)	NMR: δ ppm ($CDCl_3$) J (Hz)
5a-1	38	Oil	1490 1250	2.28 (3H, s, SMe), 3.68 (3H, s, OMe), 6.38 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-2	61	96—97 (IPE)	1485 1255	1.14 (3H, t, J=7, Me), 2.28 (3H, s, SMe), 3.88 (2H, q, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-3	65	67—68 (IPE)	1485 1255	0.92 (3H, t, J=7, Me), 1.12—1.93 (2H, m, CH_2), 2.28 (3H, s, SMe), 3.85 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-4	67	63—65 (IPE)	1485 1255	0.86 (3H, t, J=7, Me), 1.00—1.70 (4H, m, $CH_2\times 2$), 2.28 (3H, s, SMe), 3.83 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-5	56	37—39 —	1485 1250	0.86 (3H, t, J=7, Me), 1.00—1.72 (6H, m, $CH_2\times 3$), 2.28 (3H, s, SMe), 3.80 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-6	50	65—67 (IPE)	1485 1235	0.86 (3H, t, J=7, Me), 1.00—1.70 (8H, m, $CH_2\times 4$), 2.28 (3H, s, SMe), 3.80 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-7	63	67—69 (IPE)	1485 1230	0.86 (3H, t, J=7, Me), 1.00—1.70 (10H, m, $CH_2\times 5$), 2.28 (3H, s, SMe), 3.80 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.52 (7H, m, ArH+imidaH)
5a-8	68	37—38 (IPE)	1480 1230	0.86 (3H, t, J=7, Me), 1.00—1.70 (12H, m, $CH_2\times 6$), 2.28 (3H, s, SMe), 3.80 (2H, t, J=7, OCH_2), 6.34 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5a-9	46	85—87 (IPE)	1485 1240	2.27 (3H, s, SMe), 4.94 (2H, s, CH_2), 6.36 (1H, s, =CH), 6.80—7.50 (12H, m, ArH+imidaH)
5a-10	59	106—107 (IPE)	1490 1245	2.28 (3H, s, SMe), 4.95 (2H, s, CH_2), 6.36 (1H, s, =CH), 6.80—7.50 (10H, m, ArH+imidaH)
5b-1	32	Oil	1490 1250	1.31 (3H, t, J=7, SCH_2Me), 2.72 (2H, q, J=7, SCH_2), 3.68 (3H, s, OMe), 6.43 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5b-2	63	79—80 (IPE)	1485 1245	1.14 (3H, t, J=7, Me), 1.31 (3H, t, J=7, SCH_2Me), 2.72 (2H, q, SCH_2), 3.88 (2H, q, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5b-3	68	48—49 (IPE)	1490 1250	0.83 (3H, t, J=7, Me), 1.32 (3H, t, J=7, SCH_2Me), 1.20—1.70 (2H, m, CH_2), 2.72 (2H, q, J=7, SCH_2), 3.82 (2H, t, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.65 (7H, m, ArH+imidaH)
5b-4	74	33—35 —	1480 1240	0.83 (3H, t, J=7, Me), 1.00—1.70 (4H, m, $CH_2\times 2$), 1.30 (3H, t, J=7, SCH_2Me), 2.71 (2H, q, J=7, SCH_2), 3.82 (2H, t, J=7, OCH_2), 6.39 (1H, s, =CH), 6.70—7.65 (7H, m, ArH+imidaH)
5b-5	66	51—52 (IPE)	1480 1240	0.83 (3H, t, J=7, Me), 1.00—1.70 (6H, m, $CH_2\times 3$), 1.30 (3H, t, J=7, SCH_2Me), 2.70 (2H, q, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.39 (1H, s, =CH), 6.70—7.65 (7H, m, ArH+imidaH)
5b-6	70	51—53 (IPE)	1485 1245	0.83 (3H, t, J=7, Me), 1.00—1.70 (8H, m, $CH_2\times 4$), 1.32 (3H, t, J=7, SCH_2Me), 2.71 (2H, q, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.39 (1H, s, =CH), 6.70—7.65 (7H, m, ArH+imidaH)
5b-7	76	56—57 (IPE)	1490 1250	0.81 (3H, t, J=7, Me), 1.00—1.70 (10H, m, $CH_2\times 5$), 1.31 (3H, t, J=7, SCH_2Me), 2.70 (2H, q, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.65 (7H, m, ArH+imidaH)
5c-1	35	71—72 (IPE)	1490 1250	0.96 (3H, t, J=7, $S(CH_2)_2Me$), 1.17—2.00 (2H, m, CH_2), 2.64 (2H, t, J=7, SCH_2), 3.66 (3H, s, OMe), 6.38 (1H, s, =CH), 6.70—7.70 (7H, m, ArH+imidaH)
5c-2	63	68—69 (IPE)	1485 1245	0.99 (3H, t, J=7, $S(CH_2)_2Me$), 1.14 (3H, t, J=7, OCH_2Me), 1.18—2.02 (2H, m, CH_2), 2.68 (2H, t, J=7, SCH_2), 3.88 (2H, q, J=7, OCH_2), 6.38 (1H, s, =CH), 6.70—7.70 (7H, m, ArH+imidaH)
5c-3	58	Oil	1485 1245	0.70—1.90 (10H, m, Me×2+$CH_2\times 2$), 2.68 (2H, t, J=7, SCH_2), 3.78 (2H, t, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.70 (7H, m, ArH+imidaH)
5c-4	58	Oil	1485 1245	0.70—2.00 (12H, m, Me×2+$CH_2\times 3$), 2.68 (2H, t, J=7, SCH_2), 3.78 (2H, t, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.70 (7H, m, ArH+imidaH)
5c-5	65	Oil	1485 1250	0.70—2.00 (14H, m, Me×2+$CH_2\times 4$), 2.67 (2H, t, J=7, SCH_2), 3.78 (2H, t, J=7, OCH_2), 6.40 (1H, s, =CH), 6.70—7.70 (7H, m, ArH+imidaH)
5d-1	39	72—74 (IPE)	1490 1245	0.90 (3H, t, J=7, $S(CH_2)_3Me$), 1.10—1.80 (4H, m, $CH_2\times 2$), 2.68 (2H, t, J=7, SCH_2), 3.68 (3H, s, OMe), 6.41 (1H, s, =CH), 6.70—7.50 (7H, m, ArH+imidaH)
5d-2	51	Oil	1495 1250	0.70—1.80 (10H, m, Me×2+$CH_2\times 2$), 2.68 (2H, t, J=7, SCH_2), 3.86 (2H, q, J=7, OCH_2), 6.39 (1H, s, =CH), 6.70—7.60 (7H, m, ArH+imidaH)
5d-3	48	Oil	1490 1250	0.70—1.80 (12H, m, Me×2+$CH_2\times 3$), 2.68 (2H, t, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.37 (1H, s, =CH), 6.70—7.60 (7H, m, ArH+imidaH)
5d-4	57	Oil	1485 1245	0.70—1.80 (14H, m, Me×2+$CH_2\times 4$), 2.68 (2H, t, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.37 (1H, s, =CH), 6.70—7.60 (7H, m, ArH+imidaH)
5d-5	62	Oil	1490 1250	0.70—1.80 (16H, m, Me×2+$CH_2\times 5$), 2.68 (2H, t, J=7, SCH_2), 3.80 (2H, t, J=7, OCH_2), 6.38 (1H, s, =CH), 6.80—7.60 (7H, m, ArH+imidaH)
5e-1	65	Oil	1480 1230	1.12 (3H, t, J=7, Me), 3.77 (2H, q, J=7, OCH_2), 6.42 (1H, s, =CH), 6.75—7.65 (12H, m, ArH+imidaH)
5e-2	68	Oil	1480 1230	0.88 (3H, t, J=7, Me), 1.13—1.94 (2H, m, CH_2), 3.73 (2H, t, J=7, OCH_2), 6.42 (1H, s, =CH), 6.79—7.58 (12H, m, ArH+imidaH)
5e-3	67	Oil	1480 1220	0.88 (3H, t, J=7, Me), 1.02—1.90 (4H, m, $CH_2\times 2$), 3.73 (2H, t, J=7, OCH_2), 6.44 (1H, s, =CH), 6.70—7.55 (12H, m, ArH+imidaH)
5f-1	51	91—93 (IPE)	1490 1245	0.88 (3H, t, J=7, Me), 1.15—1.80 (2H, m, CH_2), 2.25 (3H, s, SMe), 3.78 (2H, t, J=7, OCH_2), 6.39 (1H, s, =CH), 6.70—7.60 (6H, m, ArH+imidaH)
5f-2	58	46—47 (IPE)	1490 1245	0.87 (3H, t, J=7, Me), 1.05—1.80 (4H, m, $CH_2\times 2$), 2.25 (3H, s, SMe), 3.78 (2H, t, J=7, OCH_2), 6.38 (1H, s, =CH), 6.70—7.60 (6H, m, ArH+imidaH)
5f-3	63	Oil	1490 1250	0.87 (3H, t, J=7, Me), 1.00—1.80 (6H, m, $CH_2\times 2$), 2.25 (3H, s, SMe), 3.78 (2H, t, J=7, OCH_2), 6.36 (1H, s, =CH), 6.70—7.60 (6H, m, ArH+imidaH)

Table V. (continued)

No.	Yield (%)	mp (°C) (Recry. solv.[a])	IR (cm^{-1}) (KBr)	NMR: δ ppm ($CDCl_3$) J (Hz)
6a-1	54	Oil	1490 1250	0.92 (3H, t, J=7, Me), 1.10—1.80 (4H, m, $CH_2\times2$), 2.32 (3H, s, SMe), 3.88 (2H, t, J=7, OCH_2), 6.74 (1H, s, =CH), 6.76—7.40 (6H, m, ArH+imidaC4, C5-H), 7.56 (1H, br s, imidaC2-H)
6a-2	49	Oil	1490 1250	0.90 (3H, t, J=7, Me), 1.10—1.80 (6H, m, $CH_2\times3$), 2.32 (3H, s, SMe), 3.86 (2H, t, J=7, OCH_2), 6.74 (1H, s, =CH), 6.76—7.40 (6H, m, ArH+imidaC4, C5-H), 7.54 (1H, br s, imidaC2-H)
6a-3	59	Oil	1490 1250	0.89 (3H, t, J=7, Me), 1.10—1.80 (8H, m, $CH_2\times4$), 2.31 (3H, s, SMe), 3.86 (2H, t, J=7, OCH_2), 6.76 (1H, s, =CH), 6.75—7.40 (6H, m, ArH+imidaC4, C5-H), 7.55 (1H, br s, imidaC2-H)
6e-1	75	96—97 (IPE)	1480 1240	1.25 (3H, t, J=7, Me), 3.87 (2H, q, J=7, OCH_2), 6.70—7.50 (12H, m, ArH+imidaC4, C5-H), 7.70 (1H, br s, imidaC2-H)
6e-2	61	75—76 (IPE)	1480 1230	0.88 (3H, t, J=7, Me), 1.22—2.00 (2H, m, CH_2), 3.86 (2H, t, J=7, OCH_2), 6.70—7.50 (12H, m, ArH+imidaC4, C5-H), 7.70 (1H, br s, imidaC2-H)
6e-3	66	97—98 (IPE)	1480 1240	0.88 (3H, t, J=7, Me), 1.08—1.80 (4H, m, $CH_2\times2$), 3.87 (2H, t, J=7, OCH_2), 6.70—7.50 (12H, m, ArH+imidaC4, C5-H), 7.70 (1H, br s, imidaC2-H)
11-1	54	Oil	1450 1240	0.90 (3H, t, J=7, Me), 1.20—1.70 (2H, m, CH_2), 3.72 (3H, s, OMe), 3.80 (2H, t, J=7, OCH_2), 6.48 (1H, s, =CH), 6.70—7.50 (6H, m, ArH+imidaC4, C5-H), 7.56 (1H, br s, imidaC2-H)
11-2	60	Oil	1450 1240	0.90 (3H, t, J=7, Me), 1.00—1.70 (4H, m, $CH_2\times2$), 3.74 (3H, s, OMe), 3.84 (2H, t, J=7, OCH_2), 6.50 (1H, s, =CH), 6.70—7.50 (6H, m, ArH+imidaC4, C5-H), 7.56 (1H, br s, imidaC2-H)
11-3	71	Oil	1450 1240	0.88 (3H, t, J=7, Me), 1.00—1.70 (6H, m, $CH_2\times3$), 3.74 (3H, s, OMe), 3.84 (2H, t, J=7, OCH_2), 6.50 (1H, s, =CH), 6.70—7.50 (6H, m, ArH+imidaC4, C5-H), 7.56 (1H, br s, imidaC2-H)
11-4	68	Oil	1450 1240	0.88 (3H, t, J=7, Me), 1.00—1.70 (8H, m, $CH_2\times4$), 3.74 (3H, s, OMe), 3.84 (2H, t, J=7, OCH_2), 6.48 (1H, s, =CH), 6.70—7.50 (6H, m, ArH+imidaC4, C5-H), 7.56 (1H, s, imidaC2-H)

a) IPE: isopropyl ether.

Table VI. Antifungal Activity of **5a**-5, Clotrimazole and Miconazole (MIC: μg/ml)

Organisms	**5a**-5		Clotrimazole[a]		Miconazole[b]	
	Without serum[c]	With 10% serum	Without serum	With 10% serum	Without serum	With 10% serum
Candida albicans NHL 4019	12.5	50	25	50	25	100
C. albicans IFO 1060	0.39	3.12	1.56	12.5	0.39	25
Trichophyton mentagrophytes QM 248	0.05	0.39	0.20	3.12	1.56	50
T. tonsurans IFO 5928	0.025	0.39	0.20	3.12	0.20	50
T. rubrum NHL J	0.025	0.20	0.39	6.25	0.20	25
Microsporum gypseum IFO 8231	0.10	3.12	0.39	6.25	6.25	50
M. audouini IFO 6074	0.05	0.39	0.20	3.12	1.56	50

a) 1-[(2-Chlorophenyl)diphenylmethyl]-1*H*-imidazole. *b*) 1-[2-(2,4-Dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1*H*-imidazole. *c*) Horse serum.

rivatives, the most active compound in each series was **5b**-5, **5c**-4 and **5d**-3, respectively. These results showed that the compounds with 6 to 7 carbons, which were the sum of the carbons of *S*-alkyl and *O*-alkyl substituents, were most active in each series of 2-alkylsulfides.

The *Z*-isomers **6a**-2 and **6a**-3 were slightly less active than corresponding *E*-isomers **5a**-5 and **5a**-6.

In the series of (*E*)-2-phenylsulfides (**5e**-1-3), **5e**-2 was most active. But it was less active than (*E*)-2-alkylsulfides (**5a**-5, **5a**-6, **5b**-4, **5c**-4 or **5d**-3), and in this case *Z*-isomers (**6e**-1-3) were also less active than *E*-isomers (**5e**-1-3). There was also a similarity observed in the case of 2-methylsulfides. Further, we compared the activities of 2-methylsulfides with chloro substituent at the 5 position of the benzene ring, (**5f**-1-3), with that of **5a**-5. In this case, **5a**-5 was also found to be most active.

Next, we compared the activities of 2-methylsulfides (**5a**) with those of 2-methoxy derivatives (**11**-1-4) and found that the **5a** series was more active than the **11** series.

In conclusion, (2-sulfinylvinyl)imidazoles showed excellent antifungal activities against dermatophytes and yeast cells, with the activities of **5a**-5 and **5a**-6 the best of all.

The antifungal activity of **5a**-5 in comparison with some known synthetic antifungal agents is shown in Table VI.[5a-e] Against dermatophytes, **5a**-5 was more active than the other antifungals, and when the agar was supplemented with 10% horse serum,[6] the activity of **5a**-5 was also more active than the others. The antifungal activity of **5a**-6 was equally as active as **5a**-5, and further experiments are ongoing.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a JASCO A-102 IR spectrometer. Proton magnetic resonance (^{1}H-NMR) was taken on a Hitachi R-24B (60 MHz) or JEOL FX90Q spectrometer (90 MHz). Chemical shifts are expressed in ppm values relative to tetramethylsilane (TMS) as an internal standard: s=singlet, d=doublet, t=triplet, m=multiplet and br=broad. Positive mass spectra and high-resolution mass spectra (MS) were taken on a JEOL JMX-DX303 mass spectrometer by a direct inlet method. Column chromatography was carried out on silica gel (Wakogel C-200). HPLC was carried out on an apparatus consisting of a Hitachi L-600 pump with a Hitachi L-400 ultraviolet (UV) detector using a Lichrosorb SI 60

column (5 μm, 4 i.d. × 250 mm) and the solvent system: chloroform–n-hexane–ethanol–acetic acid–triethylamine (1480 : 400 : 120 : 10 : 1 v/v) at a flow rate of 1.5 ml/min. The retention times of **3a** and **4a** were 5.4, 3.6 min, respectively.

Antifungal Test A susceptibility test for fungi was performed using the agar dilution test method on Sabouraud dextrose agar (SDA). Test samples, which were dissolved in 1% dimethyl sulfoxide (DMSO), were added to SDA at a final concentration range of 0.05 to 100 μg/ml. Inocula suspension, 10^6 cells/ml, was spotted on the agar surface with a multipoint inoculator. The MIC was defined as the lowest concentration of the sample preventing macroscopically visible growth after incubation at 27 °C for 3 d (for yeasts) and for 7 d (for dermatophytic fungi). The effect of serum on MIC was determined using SDA containing 10% horse serum.

(*E*)-(3a) and (*Z*)-1-[1-(2-Hydroxyphenyl)-2-(methylthio)ethenyl]-1*H*-imidazole (4a) A solution of 15% sodium methylmercaptide (5.45 ml) was added to a stirred solution of 2-bromo-2′-hydroxyacetophenone (**1a**, 2.52 g) in methanol (190 ml) in an ice bath. The reaction mixture was stirred for 30 min at room temperature. The solvent was evaporated under reduced pressure, and the residue was acidified with diluted hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with diluted hydrochloric acid and water, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was chromatographed (chloroform) to give 2′-hydroxy-2-(methylthio)acetophenone (**2a**, 2.10 g, 98%) as yellow liquid. MS m/z: 182 (M^+).

Thionyl chloride (2.06 g) was added to a stirred suspension of imidazole (4.71 g) in dichloromethane (25 ml) in an ice bath. The reaction mixture was stirred for 30 min at room temperature. Then a solution of **2a** (2.10 g) in dichloromethane (5 ml) was added to the above solution in an ice bath. The reaction mixture was stirred for 30 min at room temperature, washed with water and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was chromatographed (chloroform : methanol = 10 : 1). First, **4a** (0.32 g, 12%) was obtained as colorless needles, and secondly **3a** (1.50 g, 56%) was obtained as colorless prisms.

The series of **3b—f** and **4b—f** were prepared in the same manner. The yields were **3b**: 48%, **4b**: 10%; **3c**: 37%, **4c**: 7%; **3d**: 45%, **4d**: 8%; **3e**: 31%, **4e**: 7%; **3f**: 51%, **4f**: 12% (Table IV).

(*Z*)-1-[1-(2-Hydroxyphenyl)-2-(methylthio)ethenyl]-1*H*-imidazole (4a) **3a** (6.70 g) was suspended in diethylene glycol dimethyl ether (140 ml), and gently refluxed for about 1 h. Then the temperature was kept at 50 °C with stirring for 1 h. The precipitate was collected, washed with ether and recrystallized from methanol to give **4a** (2.27 g, 34%).

(*E*)-1-[2-(Methylthio)-1-[2-(pentyloxy)phenyl]ethenyl]-1*H*-imidazole (5a-5) A solution of **3a** (1.5 g), KOH (0.51 g) and pentyl bromide (1.15 g) in dimethylformamide (DMF) (10 ml) was stirred for 3 h at room temperature. Then the reaction mixture was poured into water and extracted with ether. The organic layer was washed with water and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was chromatographed (chloroform) to give **5a**-5 (1.10 g, 56%) as colorless crystals. MS m/z: 302 (M^+).

The series of **5a—f**, **6a, e** and **11** were also prepared in the same manner (Table V).

X-Ray Crystallography The hydrochloride salt of **5a**-5 was recrystallized from acetonitrile to give colorless columns. mp 145—147 °C. IR (KBr): 1595, 1445, 1250 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.89 (3H, t, J = 6 Hz, C–Me), 1.01—1.72 (6H, m, CH_2 × 3), 2.42 (3H, s, SMe), 3.88 (2H, t, J = 6 Hz, OCH_2), 6.95—7.80 (6H, m, ArH + imida C4, C5-H), 7.22 (1H, s, =CH), 9.01 (1H, t, J = 1 Hz, imida C2-H). *Anal.* Calcd for $C_{17}H_{22}N_2OS \cdot HCl$: C, 60.25; H, 6.84; Cl, 10.46; N, 8.27; S, 9.46. Found: C, 60.17; H, 6.78; Cl, 10.55; N, 8.25; S, 9.20.

Crystal data of this compound are listed in Table VII.

The lattice constants and intensity data were obtained on a Rigaku AFC-5 diffractometer using graphite-monochromated Cu$K_{\alpha 1}$ radiation by θ—2θ scan method. A total of 3019 reflexions were measured within the 2θ of 120°. Intensity data were corrected for Lorentz and polarization factors, but no absorption correction was applied. The crystal structure was determined by the direct method using SAPI 85[7] and refined by the full-matrix last-squares method minimizing F^2. Hydrogen atoms were located from difference Fourier map except pentyl hydrogens. The final R and R_w were 0.074 and 0.105, respectively, for 2634 reflexions with $F > F(\sigma)$, including anisotropic thermal parameters for non-hydrogen atoms and isotropic thermal parameters for hydrogen atoms. The ORTEP drawing is shown in Fig. 1.

(*E*)-1-[1-(2-Hydroxyphenyl)-2-methoxyethenyl]-1*H*-imidazole (10) A solution of Grignard reagent, prepared from 2-benzyloxy-bromobenzene

TABLE VII. Crystal Data of **5a**-5·HCl

Formula	$C_{17}H_{23}ClN_2OS$
Formula weight	338.90
Crystal size	0.85 × 0.35 × 0.35 mm
Space group	Triclinic $P\bar{1}$
Cell dimension	a = 9.729 (2) Å
	b = 13.309 (2) Å
	c = 8.703 (4) Å
	α = 95.71 (2)°
	β = 109.05 (3)°
	γ = 114.90 (1)°
	V = 928.6 (6) Å^3
	$F(000)$ = 360
Calculated density	D_C = 1.212 Mg m^{-3}
Number of formula units	Z = 2
Radiation and wavelength	$\lambda(CuK_{\alpha 1})$ = 1.5405 Å
Linear absorption coefficient	μ = 2.839 mm^{-1}

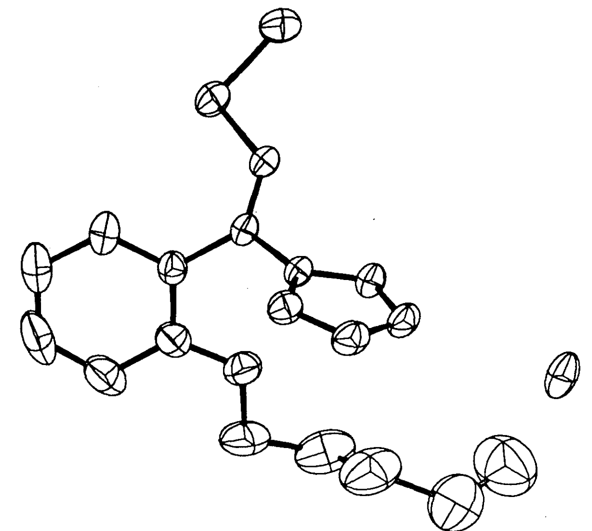

Fig. 1. ORTEP Drawing of **5a**-5·HCl

(7.90 g) and magnesium (0.73 g) in dry THF (4 ml), was added to a solution of methoxyacetonitrile (1.90 g) in dry THF (20 ml) in an ice bath. Following the addition, the solution was stirred for 2 h at room temperature and poured into 1 N hydrochloric acid. Then the mixture was washed with ether, made alkaline with diluted ammonium hydroxide and extracted with ether. After removal of the solvent, 1 N hydrochloric acid was added to the residue, and the reaction mixture was refluxed for 30 min, cooled to room temperature, and extracted with ether. The ether layer was dried over $MgSO_4$, and evaporated under reduced pressure. The residue was distilled under reduced pressure to give 2′-benzyloxy-2-methoxyacetophenone (**8**, 4.07 g, 56%) as colorless oil. bp 192—194 (2 mmHg). MS m/z: 256 (M^+). IR (NaCl): 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.45 (3H, s, OMe), 4.67 (2H, s, $COCH_2$), 5.24 (2H, s, OCH_2), 6.70—8.10 (9H, m, ArH).

The reaction mixture of **8** (0.40 g), Pd-black (0.1 g) in ethanol (30 ml) was stirred for 1 h at hydrogen atmosphere. Then the catalyst was filtered off, and the filtrate was evaporated under reduced pressure. The residue was chromatographed (chloroform), to give 2′-hydroxy-2-methoxyacetophenone (**9**, 0.25 g, 96%) as colorless oil. MS m/z: 166 (M^+). IR (NaCl): 1650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.50 (3H, s, OMe), 4.68 (2H, s, $COCH_2$), 6.69—7.77 (4H, m, ArH), 11.96 (1H, s, OH).

Thionyl chloride (0.73 g) was added to a stirred suspension of imidazole (1.67 g) in dichloromethane (10 ml) in an ice bath; then reaction mixture was stirred for 30 min at room temperature. Then a solution of **9** (0.68 g) in dichloromethane (5 ml) was added to the above solution in an ice bath. The reaction mixture was stirred for 1 h at room temperature, washed with water and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was recrystallized from MeOH to give **10** (0.63 g, 72%) as colorless needles. mp 200—203 °C (dec.). IR (KBr): 1450, 1225 cm^{-1}. ^{1}H-NMR (CD_3OD): 3.77 (3H, s, OMe), 6.59 (1H, s,

=CH), 6.68—7.35 (6H, m, ArH+imida C4, C5-H), 7.65 (1H, br s, C2-H). High resolution MS: Calcd for $C_{12}H_{12}N_2O_2$: 216.0899. Found: 216.0929.

Acknowledgement The authors are grateful to the staff members of the central analytical center of Tokyo College of Pharmacy for the elemental analysis.

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Synthesis and Antitumor Activities of Novel Benzoylphenylurea Derivatives[1)]

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Seventy novel benzoylphenylurea compounds were synthesized and their antitumor activities were examined *in vivo* against P388 leukemia. *N*-(2-Nitrobenzoyl)-*N'*-[4-(2-pyrimidinyloxy)phenyl]ureas showed the highest antitumor activities when dosed intraperitoneally or orally. Their structure–activity relationships were examined with particular focus on the position and the variety of substituent on each aryl ring.

Keywords benzoylphenylurea; *N*-(2-nitrobenzoyl)-*N'*-[4-(2-pyrimidinyloxy)phenyl]urea; *N*-[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl)urea; antitumor agent; structure–activity relationship

Benzoylphenylurea compounds are well known as insecticides which inhibit the chitin synthesis of insects, and some of them are commercially available.[2)] For more than a decade, we have synthesized benzoylphenylureas to examine their insecticidal activities.[3)] *N*-[4-(3-Chloro-5-trifluoromethyl-2-pyridyloxy)-3,5-dichlorophenyl]-*N'*-(2,6-difluorobenzoyl)urea (chlorfluazuron: Fig. 1) was selected from among those compounds and has now been commercialized. Some of its analogues were found to possess antitumor activities by random screening *in vitro* (Yoshida sarcoma). Particularly, *N*-(2-chlorobenzoyl)-*N'*-[3-chloro-4-(5-trifluoromethyl-2-pyridyloxy)phenyl]urea (**2**: Fig. 2) showed high antitumor activity *in vivo* (P388 leukemia), while chlorfluazuron itself had no antitumor activity. Since benzoylphenylureas in general are almost insoluble in water and most organic solvents, they have the disadvantage of being difficult to formulate. Judging from the high dosage (>400 mg/(kg·d)) at which compound **2** showed antitumor activity against P388 leukemia by intraperitoneal injection and the lack of antitumor activity by oral administration, the clinical use of **2** was thought to be difficult. Two methods are considered to overcome this problem. One is a chemical modification to find a compound which shows antitumor activity at lower dosage, and this paper deals with the structure–activity relationships (SAR) utilized during the designing process for a more potent compound. The other is a modification seeking compounds which have higher solubility in water or in any organic solvents, and these studies are now being conducted.

chlorfluazuron

Fig. 1

2

Fig. 2

Synthesis Benzoylphenylurea compounds can be synthesized in excellent yield by the following two synthetic routes (Chart 1). One is the synthetic route from corresponding benzoylisocyanates and aniline derivatives (method A).[4)] The other is the synthetic route from corresponding benzamides and phenylisocyanates (method B). Since method B tended to give by-products, *N*,*N'*-diphenylureas, method A was adopted in this study. The structural assignment was carried out by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and elemental analysis.

Two methods were used for preparation of aniline intermediates (Chart 2). In method C, aniline intermediates were synthesized by reaction of substituted aminophenols with arylhalides. Method D included condensation of substituted phenols and arylhalides followed by nitration and reduction. The conversion of substituents of intermediates into the desired substituents was performed by standard procedures in the course of method D. For example, aniline moieties of compounds **6**, **44** and **45** were constructed using this conversion of substituents (Chart 3).

Structure–Activity Relationships The structures, melting points, ^{1}H-NMR spectral data, synthetic methods of corresponding aniline moieties, and antitumor activities of compounds **1**—**70** are summarized in Table I. The structure–activity relationships of benzoylphenylureas, which were derived by the extended modification of original lead compound **2** putting an emphasis on changing

method A

method B

Chart 1

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method C

method D

X^1: Cl, Br

Chart 2

6

X^1: Cl, Br

X^2: CN, SCN

44: X^2=CN

45: X^2=SCN

Chart 3

substituent (X) of the benzoyl moiety, substituent (Y) of the aniline moiety, and the pyridine moiety (Z) are mentioned later. Since oral administration required a greater quantity of compound than intraperitoneal injection in order to find the optimal dose, we primarily used the results of intraperitoneal dosing for SAR. Furthermore, compounds (**2**, **3**, **17**, **23**, **57**, **61**, **66** and **67**) showed no activity with oral administration in our screening system in spite of their inherent activity by intraperitoneal injection. These facts suggest that these compounds may have low bioavailability when administered orally. The antitumor activity was defined as the dosage level at which *T*/*C* value of 150% or more was obtained[5] when SAR analysis was carried out.

Influence of Substituent (X) of Benzoyl Moiety It was found by comparison of **15**—**19** and **28** that the antitumor activity decreased in the following order with respect to the substituent at 2-position of the benzoyl moiety.

$NO_2 \gg Cl > Br > F > I, \ CF_3$

Compounds **20**, **21** and **22** bearing substituents CN, CH_3 and OCH_3, respectively, at 2-position showed no antitumor activity. Comparison of **49** and **50** with **35** indicated that 3-NO_2 compound of benzoyl moiety had no activity and that the activity of 4-NO_2 compound was inferior to that of 2-NO_2 compound. As to di-substituted compounds, comparison of **4** and **5** with **3** showed that 2,5-NO_2, Cl compound had lower activity than 2-NO_2 compound, and 2,3-NO_2, Cl compound had no activity. It was found by comparing **39** and **51** that the activity of 2,4-$(NO_2)_2$ compound was slightly lower than that of 2-NO_2 compound. On the other hand, compound **52** bearing 2,6-$(NO_2)_2$ substituents had no activity. Compounds bearing three or more substituents on the benzoyl moiety were not synthesized, because it was assumed in view of the lower activities of di-substituted than of 2-NO_2 compounds that those compounds would show decreased activities. The above results proved that 2-NO_2 compounds showed the highest activity with regard to substituent of the benzoyl moiety.

Influence of Substituent (Y) of Aniline Moiety Compounds bearing a wide variety of substituents (H, F, Cl, Br, NO_2, CF_3, CH_3, C_2H_5, CH_2OCH_3, CH_2CN, CH_2SCN, OCH_3, CO_2CH_3) at 3-position on the aniline moiety showed antitumor activities. Both compounds bearing electron-withdrawing groups and those bearing electron-donating groups showed good to excellent antitumor activities. Although the migration of substituent from 3-position to 2-position caused complete loss of activity (**39** *vs.* **53**), a small substituent like fluorine seemed to be tolerated at 2-position (**54** and **55**). 3,5-Di-substituted compounds had generally lower activities than mono-substituted compounds

TABLE I. Structures and Antitumor Activities of Benzoylphenylureas

(General structure: X-substituted phenyl (positions 2–6)–CONHCONH–phenyl (positions 2–6) bearing OZ and Y)

Compd. No.	X	Y	OZ	mp (°C)	^{1}H-NMR (DMSO-d_6) δ	Synthetic method of aniline moiety	Antitumor activity i.p.[a] Dose[c] (mg/kg)	i.p.[a] T/C (%)	p.o.[b] Dose[c] (mg/kg)	p.o.[b] T/C (%)
1	2-NO_2	3-Cl	4-O-C_6H_4-CF_3	234—235	7.07 (2H, d, J=9 Hz), 7.31 (1H, d, J=9 Hz), 7.57—7.91 (7H, m), 8.20 (1H, d, J=8 Hz), 10.33 (1H, br s), 11.36 (1H, br s)	C	400	136	NT	
2	2-Cl	3-Cl	4-O-C_5H_3N-CF_3	182—185	7.22—8.56 (10H, m), 10.45 (1H, br s), 11.09 (1H, br s)	C	500 400	251 165	1600	NA
3	2-NO_2	3-Cl	4-O-C_5H_3N-CF_3	191—194	7.33—8.28 (9H, m), 8.55 (1H, br s), 10.30 (1H, br s), 11.35 (1H, br s)	C	100	170	200—1600	NA
4	2-NO_2, 5-Cl	3-Cl	4-O-C_5H_3N-CF_3	179—181	7.32—7.37 (2H, m), 7.53 (1H, d, J=8 Hz), 7.85—7.96 (3H, m), 8.23—8.27 (2H, m), 8.85 (1H, s), 10.17 (1H, br s), 11.34 (1H, br s)	C	400	171	NT	
5	2-NO_2, 3-Cl	3-Cl	4-O-C_5H_3N-CF_3	207—209	7.24—8.66 (9H, m), 10.18 (1H, br s), 11.54 (1H, br s)	C	200—400	NA	NT	
6	2-NO_2	3-Cl	4-O-C_5H_3N-F	185—188	6.94—8.28 (10H, m), 10.17 (1H, br s), 11.18 (1H, br s)	D	50	153	400	142
7	2-NO_2	3-Cl	4-O-C_5H_3N-Cl	191—195	7.01—8.30 (10H, m), 10.26 (1H, br s), 11.15 (1H, br s)	C	100	210	NT	
8	2-NO_2	3-Cl	4-O-C_5H_3N-Br	207—208	7.07—8.43 (10H, m), 10.33 (1H, br s), 11.37 (1H, br s)	C	100	185	NT	
9	2-NO_2	3-CF_3	4-O-C_5H_3N-Cl	196—198	7.03—8.27 (10H, m), 10.36 (1H, br s), 11.32 (1H, br s)	D	25	160	800	167
10	2-NO_2	3-CH_3	4-O-C_5H_3N-NO_2	218—221	2.08 (3H, s), 6.98—8.37 (8H, m), 8.55 (1H, dd, J=9, 2 Hz), 8.98 (1H, d, J=2 Hz), 10.08 (1H, br s), 11.16 (1H, br s)	C	50	180	1600	155
11	2-NO_2	3-Cl	4-O-C_5H_3N-CF_3 (CF_3 adjacent to N)	210—212	7.36—8.22 (10H, m), 10.31 (1H, br s), 11.34 (1H, br s)	C	50	238	NT	
12	2-NO_2	3-Cl	4-O-C_5H_2N(F)-CF_3	171.5—173	6.92—8.07 (9H, m), 10.26 (1H, br s), 11.06 (1H, br s)	C	100	263	NT	
13	2-Cl	3-Cl	4-O-C_5H_2N(Cl)-CF_3	224—226	7.39—7.64 (6H, m), 7.96 (1H, d, J=3 Hz), 8.54 (1H, d, J=2 Hz), 8.62 (1H, d, J=2 Hz), 10.51 (1H, br s), 11.30 (1H, br s)	C	400	NA	NT	
14	2-Cl	3-Cl	4-O-C_5H_2N(Cl)-CF_3 (Cl adjacent to N)	194—196	7.29 (1H, d, J=9 Hz), 7.41—7.62 (6H, m), 7.99 (1H, d, J=2 Hz), 8.35 (1H, d, J=9 Hz), 10.53 (1H, br s), 11.31 (1H, br s)	C	400 200	222 157	NT	
15	2-F	3-Cl	4-O-$C_4H_2N_2$-I	199—201	7.02—7.95 (7H, m), 8.78 (2H, s), 10.43 (1H, br s), 10.97 (1H, br s)	C	200	162	NT	
16	2-Cl	3-Cl	4-O-$C_4H_2N_2$-I	219—221	7.38 (1H, d, J=8 Hz), 7.44—7.63 (5H, m), 7.93 (1H, d, J=2 Hz), 8.88 (2H, s), 10.48 (1H, br s), 11.29 (1H, br s)	C	100 50	237 189	NT	
17	2-Br	3-Cl	4-O-$C_4H_2N_2$-I	229—233	7.37—7.94 (7H, m), 8.87 (2H, s), 10.47 (1H, br s), 11.23 (1H, br s)	C	100	165	400—800	NA
18	2-I	3-Cl	4-O-$C_4H_2N_2$-I	243—246.5	7.03—7.09 (1H, m), 7.18 (1H, d, J=9 Hz), 7.28—7.38 (3H, m), 7.72—7.75 (2H, m), 8.67 (2H, s), 10.32 (1H, br s), 11.04 (1H, br s)	C	200	133	NT	
19	2-CF_3	3-Cl	4-O-$C_4H_2N_2$-I	210—211.5	7.23—8.07 (7H, m), 8.86 (2H, s), 10.40 (1H, br s), 11.33 (1H, br s)	C	100	132	NT	
20	2-CN	3-Cl	4-O-$C_4H_2N_2$-Br	226—229	7.28 (1H, d, J=9 Hz), 7.51 (1H, dd, J=9, 2 Hz), 7.77 (4H, br s), 7.93 (1H, d, J=2 Hz), 8.71 (2H, s), 10.28 (1H, br s), 11.26 (1H, br s)	C	100—400	NA	NT	

Table I. (continued)

Compd. No.	X	Y	OZ	mp (°C)	^{1}H-NMR (DMSO-d_6) δ	Synthetic method of aniline moiety	Antitumor activity i.p.[a] Dose[c] (mg/kg)	i.p.[a] T/C (%)	p.o.[b] Dose[c] (mg/kg)	p.o.[b] T/C (%)
21	2-CH_3	3-NO_2	4-O-⟨pyrimidine⟩-Br	205—208	2.41 (3H, s), 7.23—7.68 (5H, m), 8.00 (1H, dd, J=9, 3 Hz), 8.60 (1H, d, J=3 Hz), 8.85 (2H, s), 10.65 (1H, br s), 10.85 (1H, br s)	C	800	NA	NT	
22	2-OCH_3	3-NO_2	4-O-⟨pyrimidine⟩-Br	222—227	3.94 (3H, s), 6.96—8.13 (6H, m), 8.60 (1H, d, J=2 Hz), 8.84 (2H, s), 10.61 (1H, br s), 10.94 (1H, br s)	C	800	NA	NT	
23	2-NO_2	H	4-O-⟨pyrimidine⟩-I	224—228	6.92—8.31 (8H, m), 8.86 (2H, s), 10.23 (1H, br s), 11.25 (1H, br s)	C	100	154	400—1600	NA
24	2-NO_2	3-F	4-O-⟨pyrimidine⟩-Cl	209—211	7.35—8.22 (7H, m), 8.78 (2H, s), 10.33 (1H, br s), 11.36 (1H, br s)	C	25 12.5	350 160	100 50	167 150
25	2-NO_2	3-F	4-O-⟨pyrimidine⟩-Br	217—219	7.35—8.22 (7H, m), 8.83 (2H, s), 10.32 (1H, br s), 11.35 (1H, br s)	C	25 12.5	232 263	100	184
26	2-NO_2	3-Cl	4-O-⟨pyrimidine⟩-Cl	222—225	7.30—8.44 (7H, m), 8.79 (2H, s), 10.31 (1H, br s), 11.36 (1H, br s)	C	12.5	158	NT	
27	2-NO_2	3-Cl	4-O-⟨pyrimidine⟩-Br	234—236	7.32—8.40 (7H, m), 8.84 (2H, s), 10.39 (1H, br s), 11.44 (1H, br s)	C	25 12.5	168 173	400 50	210 197
28	2-NO_2	3-Cl	4-O-⟨pyrimidine⟩-I	234—236	7.43—8.28 (7H, m), 8.88 (2H, s), 10.30 (1H, br s), 11.35 (1H, br s)	C	25 12.5	230 171	NT	
29	2-NO_2	3-Cl	4-O-⟨pyrimidine⟩-CH_3	216—218	2.20 (3H, s), 7.20—8.35 (7H, m), 8.47 (2H, s), 10.29 (1H, br s), 11.33 (1H, br s)	C	50	296	NT	
30	2-NO_2	3-Br	4-O-⟨pyrimidine⟩-Cl	228—231	7.36—8.21 (7H, m), 8.78 (2H, s), 10.34 (1H, br s), 11.35 (1H, br s)	D	12.5	174	NT	
31	2-NO_2	3-NO_2	4-O-⟨pyrimidine⟩-Cl	211—213	7.57 (1H, d, J=9 Hz), 7.76—7.96 (4H, m), 8.21 (1H, d, J=8 Hz), 8.54 (1H, d, J=2 Hz), 8.78 (2H, s), 10.48 (1H, br s), 11.43 (1H, br s)	C	50	234	1600 400	219 155
32	2-NO_2	3-NO_2	4-O-⟨pyrimidine⟩-Br	222—224	7.58 (1H, d, J=9 Hz), 7.77—8.55 (6H, m), 8.84 (2H, s), 10.49 (1H, br s), 11.44 (1H, br s)	C	50 25	254 185	400	146
33	2-NO_2	3-NO_2	4-O-⟨pyrimidine⟩-I	232—235	7.57 (1H, d, J=9 Hz), 7.76—7.97 (4H, m), 8.21 (1H, d, J=8 Hz), 8.55 (1H, d, J=2 Hz), 8.78 (2H, s), 10.48 (1H, br s), 11.44 (1H, br s)	C	50	211	NT	
34	2-NO_2	3-CF_3	4-O-⟨pyrimidine⟩	235—236	7.17—8.17 (8H, m), 8.51 (2H, d, J=6 Hz), 10.24 (1H, br s), 11.22 (1H, br s)	D	100	242	800 400	228 174
35	2-NO_2	3-CF_3	4-O-⟨pyrimidine⟩-Cl	201—205	7.30—8.27 (7H, m), 8.70 (2H, s), 10.31 (1H, br s), 11.21 (1H, br s)	D	3.125	160	6.25	237
36	2-NO_2	3-CF_3	4-O-⟨pyrimidine⟩-Br	196—197	7.36—8.43 (7H, m), 8.86 (2H, s), 10.42 (1H, br s), 11.36 (1H, br s)	D	3.125	153	6.25	186
37	2-NO_2	3-CF_3	4-O-⟨pyrimidine⟩-I	211—214	7.46 (1H, d, J=9 Hz), 7.76—8.22 (6H, m), 8.87 (2H, s), 10.40 (1H, br s), 11.38 (1H, br s)	D	12.5	167	NT	
38	2-NO_2	3-CH_3	4-O-⟨pyrimidine⟩-Cl	204—206	2.06 (3H, s), 6.97—8.30 (7H, m), 8.67 (2H, s), 10.17 (1H, br s), 11.21 (1H, br s)	C	3.125	153	25 12.5	205 184
39	2-NO_2	3-CH_3	4-O-⟨pyrimidine⟩-Br	214—217	2.09 (3H, s), 6.98—8.37 (7H, m), 8.78 (2H, s), 10.23 (1H, br s), 11.30 (1H, br s)	C	3.125	163	25	204
40	2-NO_2	3-CH_3	4-O-⟨pyrimidine⟩-CH_3	227—229	2.05 (3H, s), 2.18 (3H, s), 6.96—8.40 (7H, m), 8.49 (2H, s), 10.25 (1H, br s), 11.31 (1H, br s)	C	200 50	339 210	NT	

Table I. (continued)

Compd. No.	X	Y	OZ	mp (°C)	^{1}H-NMR (DMSO-d_6) δ	Synthetic method of aniline moiety	Antitumor activity i.p.[a] Dose[c] (mg/kg)	i.p.[a] T/C (%)	p.o.[b] Dose[c] (mg/kg)	p.o.[b] T/C (%)
41	2-NO_2	3-C_2H_5	4-O-[pyrimidine]-Cl	181—184	1.12 (3H, t, J=8 Hz), 2.59 (2H, q, J=8 Hz), 7.00—8.36 (7H, m), 8.70 (2H, s), 10.23 (1H, br s), 11.28 (1H, br s)	D	12.5 3.125	343 157	25 6.25	209 154
42	2-NO_2	3-C_2H_5	4-O-[pyrimidine]-Br	211—212.5	1.10 (3H, t, J=8 Hz), 2.50 (2H, q, J=8 Hz), 6.99—8.30 (7H, m), 8.74 (2H, s), 10.16 (1H, br s), 11.20 (1H, br s)	D	12.5 3.125	232 157	50 25	216 172
43	2-NO_2	3-CH_2OCH_3	4-O-[pyrimidine]-Br	187—189	3.10 (3H, s), 4.21 (2H, s), 7.10 (1H, d, J=9 Hz), 7.93—8.23 (6H, m), 8.67 (2H, s), 10.16 (1H, br s), 11.15 (1H, br s)	C	100 50	250 174	800	214
44	2-NO_2	3-CH_2CN	4-O-[pyrimidine]-Br	231—233	3.87 (2H, s), 7.10—8.30 (7H, m), 8.73 (2H, s), 10.20 (1H, br s), 11.17 (1H, br s)	D	200 100	237 205	NT	
45	2-NO_2	3-CH_2SCN	4-O-[pyrimidine]-Br	189—190	3.53 (2H, s), 7.13—8.30 (7H, m), 8.70 (2H, s), 10.20 (1H, br s), 11.20 (1H, br s)	D	200 100	207 158	1600 800	168 155
46	2-NO_2	3-$CH_2CO_2CH_3$	4-O-[pyrimidine]-Br	191—192	3.60 (3H, s), 3.70 (2H, s), 7.30—8.47 (7H, m), 8.80 (2H, s), 10.27 (1H, br s), 11.30 (1H, br s)	D	100—400	NA	200—1600	NA
47	2-NO_2	3-OCH_3	4-O-[pyrimidine]-Cl	219—221	3.71 (3H, s), 7.20—8.30 (7H, m), 8.63 (2H, s), 10.20 (1H, br s), 11.17 (1H, br s)	C	200 50	189 150	1600	180
48	2-NO_2	3-CO_2CH_3	4-O-[pyrimidine]-Br	200—201	3.63 (3H, s), 7.35 (1H, d, J=9 Hz), 7.60—8.40 (6H, m), 8.77 (2H, s), 10.37 (1H, br s), 11.33 (1H, br s)	D	400	156	NT	
49	3-NO_2	3-CF_3	4-O-[pyrimidine]-Cl	238—243	7.45 (1H, d, J=9 Hz), 7.63—8.85 (8H, m), 10.76 (1H, br s), 11.42 (1H, br s)	D	800	NA	NT	
50	4-NO_2	3-CF_3	4-O-[pyrimidine]-Cl	234—236	7.47 (1H, d, J=9 Hz), 7.76—8.49 (6H, m), 8.76 (2H, s), 10.76 (1H, br s), 11.41 (1H, br s)	D	100 50	209 184	NT	
51	2,4-$(NO_2)_2$	3-CH_3	4-O-[pyrimidine]-Br	232—235	2.06 (3H, s), 7.07 (1H, d, J=9 Hz), 7.22—7.50 (2H, m), 8.02 (1H, d, J=8 Hz), 8.52—8.86 (4H, m), 9.82 (1H, br s), 11.20 (1H, br s)	C	12.5	184	50	226
52	2,6-$(NO_2)_2$	3-Cl	4-O-[pyrimidine]-Br	169—171	7.09 (1H, d, J=9 Hz), 7.70—8.82 (3H, m), 8.67 (2H, d, J=9 Hz), 8.80 (2H, s), 11.12 (1H, br s), 12.66 (1H, br s)	C	100—400	NA	100—800	NA
53	2-NO_2	2-CH_3	4-O-[pyrimidine]-Br	219—221.5	2.30 (3H, s), 6.60—8.06 (7H, m), 8.47 (2H, s), 9.87 (1H, br s), 11.41 (1H, br s)	C	100—400	NA	NT	
54	2-NO_2	2-F, 3-Cl	4-O-[pyrimidine]-Br	201—202.5	7.39 (1H, dd, J=9, 2 Hz), 7.74—8.46 (5H, m), 8.91 (2H, s), 10.52 (1H, br s), 11.59 (1H, br s)	C	400	151	NT	
55	2-NO_2	3-Cl, 6-F	4-O-[pyrimidine]-Br	226—229	7.55—8.40 (6H, m), 8.84 (2H, s), 10.52 (1H, br s), 11.55 (1H, br s)	C	25	189	NT	
56	2-NO_2	3-Cl, 5-CF_3	4-O-[pyrimidine]-Br	221—224	7.58—8.35 (6H, m), 8.85 (2H, s), 10.47 (1H, br s), 11.47 (1H, br s)	C	50	255	NT	
57	2-NO_2	3-Cl, 5-CH_2OCH_3	4-O-[pyrimidine]-Br	236—238	3.20 (3H, s), 4.36 (2H, s), 7.49—8.41 (6H, m), 8.86 (2H, s), 10.32 (1H, br s), 11.36 (1H, br s)	C	200	228	200—1600	NA
58	2-NO_2	2-CH_3	3-O-[pyrimidine]-Br	202—206	2.06 (3H, s), 6.80—8.31 (7H, m), 8.73 (2H, s), 10.25 (1H, br s), 11.38 (1H, br s)	C	100	126	NT	
59	2-NO_2	4-CH_3	3-O-[pyrimidine]-Cl	214—219	2.04 (3H, s), 7.30—8.33 (7H, m), 8.71 (2H, s), 10.18 (1H, br s), 11.19 (1H, br s)	D	25 12.5	188 167	100 50	190 167

TABLE I. (continued)

Compd. No.	X	Y	OZ	mp (°C)	^{1}H-NMR (DMSO-d_6) δ	Synthetic method of aniline moiety	Antitumor activity i.p.[a) Dose[c) (mg/kg)	i.p.[a) T/C (%)	p.o.[b) Dose[c) (mg/kg)	p.o.[b) T/C (%)
60	2-NO_2	4-C_2H_5	3-O-(N ring N)-Br	223—227	1.10 (3H, t, J=8 Hz), 2.47 (2H, q, J=8 Hz), 7.25—8.36 (7H, m), 8.77 (2H, s), 10.20 (1H, br s), 11.19 (1H, br s)	D	50 25	226 174	400 200	214 176
61	2-NO_2	H	4-O-(N−N ring)-Br	219—222	7.18—8.31 (10H, m), 10.28 (1H, br s), 11.27 (1H, br s)	C	100	266	200—800	NA
62	2-NO_2	3-F	4-O-(N−N ring)-Br	221—224	7.38—8.21 (9H, m), 10.37 (1H, br s), 11.37 (1H, br s)	D	25	208	400 200	157 152
63	2-NO_2	3-Cl	4-O-(N−N ring)-Cl	240—242	7.45—8.33 (9H, m), 10.32 (1H, br s), 11.33 (1H, br s)	C	200	171	NT	
64	2-NO_2	3-Cl	4-O-(N−N ring)-CF_3	226—230	7.56—8.40 (9H, m), 10.40 (1H, br s), 11.53 (1H, br s)	C	100	146	NT	
65	2-NO_2	3-Cl	4-O-(N−N ring)-$CO_2C_2H_5$	208—210	1.33 (3H, t, J=7 Hz), 4.38 (2H, q, J=7 Hz), 7.50 (1H, d, J=9 Hz), 7.59—7.97 (6H, m), 8.21 (1H, d, J=8 Hz), 8.27 (1H, d, J=9 Hz), 10.36 (1H, br s), 11.38 (1H, br s)	C	100—400	NA	NT	
66	2-NO_2	3-Br	4-O-(N−N ring)-Br	241—243	7.57—8.37 (9H, m), 10.37 (1H, br s), 11.35 (1H, br s)	D	400 100	161 151	100—800	NA
67	2-NO_2	3-NO_3	4-O-(N−N ring)-Br	222—225	7.58—8.56 (9H, m), 10.51 (1H, br s), 11.45 (1H, br s)	C	100 25	292 161	200—800	NA
68	2-NO_2	3-CF_3	4-O-(N−N ring)-Cl	212—215	7.30—8.34 (9H, m), 10.38 (1H, br s), 11.33 (1H, br s)	D	25 12.5	227 171	25	126
69	2-NO_2	3-Cl	4-O-(N, S ring; Cl, Cl)	209—211	7.60—8.28 (7H, m), 10.33 (1H, br s), 11.40 (1H, br s)	C	400	182	NT	
70	2-NO_2	3-Cl	4-O-(N, S fused benzo ring)	203—206	7.31—7.99 (10H, m), 8.21 (1H, d, J=8 Hz), 10.37 (1H, br s), 11.39 (1H, br s)	C	400	162	NT	

a) Intraperitoneal injection. *b)* *Per os* (oral) administration. *c)* When two values are listed, the upper one is the optimum dose at which the maximum *T/C* value is shown and the lower one is the minimum dose at which *T/C* value is 150% or more. When the optimum dose equals the minimum dose or when the maximum *T/C* value is between 125% and 150%, only one value is listed. When the compound shows no significant activity (NA; $T/C < 125\%$), the range of examined dose is listed. NT: not tested.

at 3-position (for example, see the antitumor activities of **27**, **43** and **57**). It is obvious from these results that the introduction of three or more substituents into the aniline moiety probably results in a reduction of antitumor activity. Thus, mono-substituted compounds at 3-position showed the highest activity so far as substituent of the aniline moiety. Specifically, CH_3, C_2H_5 and CF_3 gave the highest activity, and the second group with excellent activity was compounds substituted with F, Cl or Br.

Influence of (Hetero)aryl Moiety (Z) From comparison of the antitumor activities of a set of compounds which have the same substituents X and Y, and different (hetero)aryl ring bearing the same substituent thereon (for example, between **1** and **3** or among **9**, **35** and **68**, *etc.*), it was found that the order of antitumor activities of the compounds decreased as follows.

pyrimidine > pyridazine ≧ pyridine > benzene

Among the three modifying parts (X, Y, Z), the greatest flexibility for structural changes was found in (hetero)aryl ring. Comparison of antitumor activities of **11** with **3** indicated that the migration of substituent from 5-position

CONHCONH O N N R_2 NO_2 R^1

Fig. 3

to 6-position on pyridine ring did not influence antitumor activity. Furthermore, some compounds bearing two substituents on pyridine ring, such as **12** and **14**, showed slightly higher antitumor activities than the corresponding mono-substituted compounds **3** and **2**, respectively. In contrast to *ortho* fluorinated compound to the ether bond (**12**), *ortho* chlorinated compound (**13**) did not show antitumor activity. There is thought to be a limitation on size at *ortho* position to the ether bond on (hetero)aryl ring. Although compounds substituted at 4-position on pyrimidine ring and compounds bearing two or three substituents on pyrimidine ring were not synthesized, those compounds are estimated to show good antitumor activities in view of SAR for substituents on pyridine ring. Compounds **59** and **60**, which are considered to be obtained by exchanging methyl and ethyl groups with the

pyrimidinyloxy substituents originally existing at 3 and 4-positions of aniline moiety of **38** and **42**, respectively, had high activities, although relatively lower than those of **38** and **42**. Although two additional compounds, **69** and **70**, which have thiazole or benzothiazole ring, showed moderate antitumor activities, no highly active compound like pyrimidinyloxy derivatives could be found.

In conclusion, we can say that compounds represented by Fig. 3 show the highest antitumor activity. Among them, *N*-[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl)urea (**27**: coded HO-221) has now been selected for further preclinical and clinical evaluation because of its antitumor activity even at low dosage, its effectiveness by oral administration and the ease of synthesis.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a JEOL JNM-PMX60$_{SI}$ or JEOL JNM-GSX400 spectrometer with tetramethylsilane as an internal standard, and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; br, broad.

Preparation Example of Benzoylphenylureas: *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl)urea (27) A solution of 5-bromo-2-chloropyrimidine (7.00 g, 36 mmol), 4-amino-2-chlorophenol (5.19 g, 36 mmol), K_2CO_3 (9.98 g, 72 mmol) in dimethyl sulfoxide (DMSO) (70 ml) was stirred at 120 °C for 1.5 h under N_2. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with water and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane: EtOAc = 7 : 3) to give 4-(5-bromo-2-pyrimidinyloxy)-3-chloroaniline (6.80 g, 62%), mp 86—89 °C.

A solution of 2-nitrobenzoyl isocyanate (5.76 g, 30 mmol) in 1,4-dioxane (30 ml) was added dropwise to a solution of 4-(5-bromo-2-pyrimidinyloxy)-3-chloroaniline (6.80 g, 23 mmol) in 1,4-dioxane (30 ml) with stirring at room temperature. The reaction mixture was stirred at room temperature for 9 h, then poured into water. The precipitated product was collected by filtration and washed with hot water, then MeOH to give **27** (9.42 g, 85%) as a white powder, mp 234—236 °C. *Anal.* Calcd for $C_{18}H_{11}BrClN_5O_5$: C, 43.38; H, 2.25; Br, 16.22; Cl, 7.20; N, 14.22; O, 16.24. Found: C, 43.80; H, 2.05; Br, 16.17; Cl, 7.18; N, 14.25; O, 16.40. ^{1}H-NMR (DMSO-d_6) δ: 7.32—8.40 (7H, m), 8.84 (2H, s), 10.39 (1H, br s), 11.44 (1H, br s).

Preparation of *N*-[4-(5-Fluoro-2-pyridyloxy)-3-chlorophenyl]-*N'*-(2-nitrobenzoyl)urea (6) A mixture of sodium 2-chlorophenolate (26.63 g, 0.18 mol) and 2-chloro-5-nitropyridine (25.00 g, 0.16 mol) in *N*,*N*-dimethylformamide (DMF) (100 ml) was stirred at 120 °C for 13 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 15 : 1) to give 2-(2-chlorophenoxy)-5-nitropyridine (29.63 g, 75%), mp 76—77 °C.

Iron powder (21.50 g, 0.38 mol) was gradually added to a solution of 2-(2-chlorophenoxy)-5-nitropyridine (19.25 g, 77 mmol) in AcOH (75 ml) at 80 °C. The reaction mixture was heated under reflux for 10 min, and then allowed to cool to room temperature. The resulting precipitates were removed by filtration. The filtrate was poured into water and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 3 : 2) to give 5-amino-2-(2-chlorophenoxy)pyridine (10.70 g, 63%), mp 84—85.5 °C.

A solution of $NaNO_2$ (2.60 g, 38 mmol) in water (5 ml) was added dropwise to a mixture of 5-amino-2-(2-chlorophenoxy)pyridine (8.30 g, 38 mmol) and 42% fluoboric acid (58 ml) with stirring at 0 °C. The mixture was stirred at 0 °C for 10 min, and the precipitated product was collected by filtration, dried *in vacuo* at room temperature, and then pyrolyzed at 130 °C for 10 min. The reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with water, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 9 : 1) to give 2-(2-chlorophenoxy)-5-fluoropyridine (4.47 g, 53%) as a colorless oil.

A solution of 60% HNO_3 (1.5 ml) and concentrated H_2SO_4 (3 ml) was added dropwise to a solution of 2-(2-chlorophenoxy)-5-fluoropyridine (4.47 g, 20 mmol) in concentrated H_2SO_4 (25 ml) with stirring at room temperature. The reaction mixture was stirred at room temperature for 10 min, poured into ice-water, and then extracted with EtOAc. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and evaporated. The residue was subjected to column chromatography on silica gel (*n*-hexane : EtOAc = 9 : 1). The first eluate gave 2-(2-chloro-5-nitrophenoxy)-5-fluoropyridine (0.87 g, 16%), mp 97—102 °C, and the second gave 2-(2-chloro-4-nitrophenoxy)-5-fluoropyridine (2.70 g, 50%) as a colorless oil.

Iron powder (2.50 g, 45 mmol) was gradually added to a solution of 2-(2-chloro-4-nitrophenoxy)-5-fluoropyridine (2.40 g, 8.9 mmol) in AcOH (20 ml) at 90 °C. The reaction mixture was heated under reflux for 10 min, and then allowed to cool to room temperature. The resulting precipitates were removed by filtration. The filtrate was poured into water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 3 : 2) to give 3-chloro-4-(5-fluoro-2-pyridyloxy)aniline (1.81 g, 85%), mp 107—108.5 °C.

3-Chloro-4-(5-fluoro-2-pyridyloxy)aniline (1.18 g, 7.1 mmol) was treated with 2-nitrobenzoyl isocyanate (1.37 g, 7.1 mmol) in the same way as described for **27** to give **6** (1.95 g, 83%) as a white powder, mp 231—233 °C. *Anal.* Calcd for $C_{19}H_{12}ClFN_4O_5$: C, 52.97; H, 2.81; N, 13.01. Found: C, 52.90; H, 2.79; N, 12.69. ^{1}H-NMR (DMSO-d_6) δ: 6.94—8.28 (10H, m), 10.17 (1H, br s), 11.18 (1H, br s).

Preparation of *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-cyanomethyl]-*N'*-(2-nitrobenzoyl)urea (44) A mixture of 5-bromo-2-chloropyrimidine (5.00 g, 26 mmol), *o*-cresol (3.10 g, 29 mmol), K_2CO_3 (4.30 g, 31 mmol) and DMSO (50 ml) was stirred at 100 °C for 1 h. The reaction mixture was poured into water and extracted with Et_2O. The organic layer was washed with water, dried over Na_2SO_4 and evaporated to yield 5-bromo-2-(2-methylphenoxy)-pyrimidine (6.00 g, 88%).

A mixture of 5-bromo-2-(2-methylphenoxy)pyrimidine (5.00 g, 19 mmol), *N*-chlorosuccinimide (NCS) (2.10 g, 16 mmol), benzoyl peroxide (0.80 g, 3 mmol) and CCl_4 (100 ml) was heated under reflux for 14 h, and then allowed to cool to room temperature. The separated solid was filtered off and the filtrate was evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 3 : 1) to give 5-bromo-2-(2-chloromethylphenoxy)pyrimidine (3.50 g, 74%).

A solution of 5-bromo-2-(2-chloromethylphenoxy)pyrimidine (3.50 g, 12 mmol) in DMSO (5 ml) was added dropwise to a solution of NaCN (0.80 g, 16 mmol) in DMSO (15 ml) with stirring at room temperature. The reaction mixture was stirred at room temperature for 1 h, poured into water, and then extracted with Et_2O. The organic layer was washed with 6 N HCl and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 5 : 1) to give 2-(5-bromo-2-pyrimidinyloxy)benzyl cyanide (2.40 g, 71%).

A solution of 60% HNO_3 (0.7 ml) and concentrated H_2SO_4 (0.7 ml) was added dropwise to a solution of 2-(5-bromo-2-pyrimidinyloxy)benzyl cyanide (2.30 g, 7.9 mmol) in concentrated H_2SO_4 (20 ml) with stirring at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h, poured into ice-water, and then extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 3 : 1) to give 2-(5-bromo-2-pyrimidinyloxy)-5-nitrobenzyl cyanide (1.40 g, 53%).

Iron powder (1.20 g, 21 mmol) was gradually added to a solution of 2-(5-bromo-2-pyrimidinyloxy)-5-nitrobenzyl cyanide (1.40 g, 4.2 mmol) in AcOH (30 ml) at 90 °C. The reaction mixture was heated under reflux for 20 min, and then allowed to cool to room temperature. The resulting precipitates were removed by filtration. The filtrate was poured into water and extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 2 : 1) to give 5-amino-2-(5-bromo-2-pyrimidinyloxy)-benzyl cyanide (0.76 g, 60%).

5-Amino-2-(5-bromo-2-pyrimidinyloxy)benzyl cyanide (0.70 g, 2.3 mmol) was treated with 2-nitrobenzoyl isocyanate (0.40 g, 2.4 mmol) in the same way as described for **27** to give **44** (0.85 g, 74%) as a white powder, mp 231—233 °C. *Anal.* Calcd for $C_{20}H_{13}BrN_6O_5$: C, 48.31; H, 2.63; N, 16.90. Found: C, 48.52; H, 2.41; N, 17.20. ^{1}H-NMR (DMSO-d_6) δ: 3.87 (2H, s), 7.10—8.30 (7H, m), 8.73 (2H, s), 10.20 (1H, br s), 11.17 (1H, br s).

Preparation of *N*-[4-(5-Bromo-2-pyrimidinyloxy)-3-thiocyanatomethyl]-*N'*-(2-nitrobenzoyl)urea (45) A mixture of 5-bromo-2-(2-methylpheno-

xy)pyrimidine (9.17 g, 35 mmol), *N*-bromosuccinimide (NBS) (5.10 g, 29 mmol), benzoyl peroxide (1.50 g, 6.2 mmol) and CCl_4 (100 ml) was heated under reflux for 3.5 h, and then allowed to cool to room temperature. The separated solid was filtered off and the filtrate was evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 8 : 1) to give 5-bromo-2-(2-bromomethylphenoxy)pyrimidine (9.10 g, 92%).

A solution of 5-bromo-2-(2-bromomethylphenoxy)pyrimidine (9.10 g, 27 mmol) in EtOH (50 ml) was added dropwise to a solution of KSCN (2.80 g, 29 mmol) in EtOH (50 ml) under reflux. The reaction mixture was heated under reflux for 2 h, and then allowed to cool to room temperature. The separated solid was filtered off and the filtrate was evaporated. The residue was poured into water and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and evaporated to yield 2-(5-bromo-2-pyrimidinyloxy)benzyl thiocyanate (6.60 g, 77%).

A solution of 60% HNO_3 (2.0 ml) and concentrated H_2SO_4 (2.0 ml) was added dropwise to a solution of 2-(5-bromo-2-pyrimidinyloxy)benzyl thiocyanate (6.60 g, 21 mmol) in concentrated H_2SO_4 (40 ml) with stirring at 0 °C. The reaction mixture was stirred at 0 °C for 5 min, poured into ice-water, and then extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 3 : 1) to give 2-(5-bromo-2-pyrimidinyloxy)-5-nitrobenzyl thiocyanate (2.50 g, 33%).

Iron powder (1.90 g, 34 mmol) was gradually added to a solution of 2-(5-bromo-2-pyrimidinyloxy)-5-nitrobenzyl thiocyanate (2.50 g, 6.8 mmol) in AcOH (100 ml) at 90 °C. The reaction mixture was heated under reflux for 10 min, and then allowed to cool to room temperature. The resulting precipitates were removed by filtration. The filtrate was poured into water and extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography on silica gel (*n*-hexane : EtOAc = 2 : 1) to give 5-amino-2-(5-bromo-2-pyrimidinyloxy)benzyl thiocyanate (1.40 g, 61%).

5-Amino-2-(5-bromo-2-pyrimidinyloxy)benzyl thiocyanate (1.00 g, 3.0 mmol) was treated with 2-nitrobenzoyl isocyanate (0.58 g, 3.0 mmol) in the same way as described for **27** to give **45** (1.35 g, 86%) as a white powder, mp 189—190 °C. *Anal.* Calcd for $C_{20}H_{13}BrN_6O_5S$: C, 45.38; H, 2.47; N, 15.88. Found: C, 45.42; H, 2.25; N, 15.65. ^{1}H-NMR (DMSO-d_6) δ: 3.53 (2H, s), 7.13—8.30 (7H, m), 8.70 (2H, s), 10.20 (1H, br s), 11.20 (1H, br s).

Formulation Ten parts by weight of the benzoylphenylurea compounds were added to a solution containing 1.5 parts by weight of oxyethylated polyarylphenol phosphate neutralized with triethanolamine (Soprophor FL, Rhone-Poulenc), 0.2 part by weight of silicone antifoam (SAG-471, Nuc, Silicones) and 0.3 part by weight of polyoxyethylenepolyoxypropylene block polymer in 81 parts by weight of physiological saline, and the mixture was pulverized in a wet system in a sand mill using glass beads (90% of particles having a particle size of not larger than 2 μm). Then, 7 parts by weight of xanthane gum (2% solution) was added to the mixture to give an aqueous suspension, and this suspension was diluted to the proper concentration by physiological saline.

Biological Testing Method Antitumor activities were tested by means of the protocols used for routine screening at the National Cancer Institute (Bethesda, Md.). To BDF_1 mice, P388 leukemia cells were intraperitoneally inoculated in an amount of 1×10^6 cells/mouse. A formulated compound was intraperitoneally or orally administered to mice on days 1 and 4 after the inoculation. Groups of five mice per dose level of the test compound were used with one control group of five mice. The mice were observed for 30 d for survival or death. Antitumor activity of compounds was expressed as follows:

$$\frac{\text{median survival time of treated group}}{\text{median survival time of control}} \times 100 \ (T/C)$$

Median survival times of the control group ranged from 8.7 to 10.6 d. Samples with T/C values that exceeded 125% were evaluated as antitumor-active.

Acknowledgments The authors are grateful to Dr. R. Nishiyama, former director of their laboratory, for his supervision and continued guidance throughout this work. They also express their thanks to Mr. K. Fujikawa for his helpful advice, to Mr. H. Sugi for the biological assay, and to Mr. Y. Nakamura for his experimental cooperation in a part of this work.

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Studies on Analgesic Oligopeptides. VII.[1a,2] Solid Phase Synthesis and Biological Properties of Tyr–D-Arg–Phe–βAla–NH_2 and Its Fluorinated Aromatic Amino Acid Derivatives

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Tyr–D-Arg–Phe–βAla–NH_2 (I) and its six fluorinated analogs were synthesized. Their opioid receptor binding properties were examined *in vitro* and their analgesic activity *in vivo* using the mouse writhing test. It was found that I was one of the most selective and potent μ-receptor agonists reported to date. [Tyr(2F)^1](VI) and [Tyr(3F)^1](V) derivatives of I showed similar biological properties to those of I. Since these peptides resist enzymatic degradation, it is expected that they are excellent reagents for the studies of function of μ-receptor-mediated biological properties *in vivo* and *in vitro*.

Keywords solid phase synthesis; dermorphin analog; fluorinated aromatic amino acid; opioid receptor binding assay; mouse writhing test; high μ-affinity; high μ-selectivity; analgesics

Introduction

We have studied extensively the structure–activity relationships of [D-Arg^2]dermorphin analogs.[1] In a previous study on structure–opioid activity relationships of Tyr–D-Arg–Phe–NHR, we revealed that the introduction of an acid–amide group at the alkyl moiety (R) increases μ-opioid receptor selectivity and affinity compared to its acid derivative.[1a]

In this study, a potent analgesic tetrapeptide, Tyr–D-Arg–Phe–βAla, found in our laboratory,[1c] was selected as lead peptide and the tetrapeptide amide, Tyr–D-Arg–Phe–βAla–NH_2 (I) was synthesized by the solid phase method. I showed very high selectivity for μ-receptors, as expected (Table I), as well as analgesic activity (Table II).

On the other hand, in a previous study of δ-selective [D-Ala^2, D-Leu^5]enkephalin (DADLE) analogs containing a fluorinated aromatic amino acid, it was revealed that the fluorinated derivatives did not show a dramatic change in receptor selectivity in general.[3] In studies of fluorinated drug design,[4] it is also interesting that biologically active ^{19}F compounds are good reagents for the study of receptors,[5] and ^{18}F-containing compounds are good reagents for positron emission tomography (PET).[6] A direct fluorination method of Tyr–D-Ala–Phe–Gly–NH_2 to give Tyr(3F)–D-Ala–Phe–Gly–NH_2 in high yield has recently been developed.[7] The work suggests the usefulness of Tyr-containing peptides for PET.

In this study, six kinds of I analogs (II—VII) containing fluorinated aromatic amino acid(s) were synthesized using the solid phase method. Thus, [Phe(2F)^3](II), [Phe(3F)^3](III), [Phe(4F)^3](IV), [Tyr(3F)^1](V), [Tyr(2F)^1](VI) and [Tyr(3F)^1, Phe(4F)^3)](VII) derivatives of I were obtained and their biological properties examined. V and VI showed similar biological properties to I (Tables I and II).

Results and Discussion

Peptides were synthesized by the solid phase method starting with Boc–βAla–benzhydrylamine resin as described previously.[3] After cleavage of the peptide from resin and deprotection by treatment with an HF–anisole mixture, the peptide was purified by using a medium-pressured HPLC. Homogeneity of the peptides was checked by analytical HPLC, TLC and amino acid analysis after 6 N HCl hydrolysis. Analytical data of peptides is shown in Table III.

Opioid receptor binding properties of the peptides were compared with those of DAGO,[8] DPDPE[9] and U-69593,[10] which are at present used routinely as one of the most selective ligands for μ, δ and κ-receptors, respectively, and the results are shown in Table I. Analogs synthesized in this study showed very weak κ-affinity, less than that of DAGO. The only exception is II which showed κ-affinity equi-potent to DAGO. The δ/μ selectivity ratio of 12952 of I makes compound (I) one of the most selective and potent μ-receptor agonists reported to date.[11] Among the fluorinated Phe^3 analogs of I, IV showed a marked decrease in both μ and δ-affinities, while II and III showed

TABLE I. Opioid Receptor Binding Assay of Synthetic Dermorphin Analogs

Compound	[^{3}H]DAGO (μ) K_i (nM) (±S.E.)	[^{3}H] DPDPE (δ) K_i (nM) (±S.E.)	$K_i(\delta)/K_i(\mu)$	[^{3}H]U-69593 (κ) K_i (nM) (±S.E.)
DAGO	0.37 (0.03)	175 (16)	473	360 (80)
DPDPE	321 (54)	0.461 (0.067)	0.0014	—
U-69593	—	—	—	0.49 (0.34)
Tyr–D-Arg–Phe–βAla	0.020 (0.018)	42 (29)	2100	—[a]
Tyr–D-Arg–Phe–βAla–NH_2 (I)	0.021 (0.004)	272 (172)	12952	1365 (182)
[Phe(2F)^3] I (II)	0.144 (0.062)	656 (274)	4556	1292 (332)
[Phe(3F)^3] I (III)	0.082 (0.013)	353 (151)	4305	477 (167)
[Phe(4F)^3] I (IV)	0.87 (0.26)	2546 (1537)	2926	1079 (535)
[Tyr(3F)^1] I (V)	0.038 (0.008)	391 (214)	10289	1598 (162)
[Tyr(2F)^1] I (VI)	0.109 (0.037)	1060 (653)	9725	2375<
[Tyr(3F)^1, Phe(4F)^3] I (VII)	3.6 (1.4)	2571 (2183)	714	2375<

a) IC_{50} 10000< (see ref. 1*a*).

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relatively little decrease at both receptors, indicating that the fluorination at position 4 of the Phe^3 ring is more effective for low affinity for μ and δ-receptors than that at position 2 or 3. On the other hand, the $Tyr(3F)^1$ analog, V, showed a high μ-affinity and selectivity nearly comparable to I.

The $Tyr(2F)^1$ analog, VI, also showed half the potency of V without a change in μ-selectivity. A multi-fluorinated analog, VII, resulted in a great decrease in μ-affinity, possibly due to $Phe(4F)^3$ residue as described above. In general, μ-selective I analogs containing fluorinated aromatic amino acids did not dramatically change receptor affinities as did the DADLE analogs described previously.[3] Incubation of all synthetic analogs with rat brain homogenate at 37 °C for 5 h produced no detectable degradation product on HPLC, showing good stability of these peptides against degradation enzymes in the brain. These results indicate that the data of the *in vitro* binding assay described above is reliable.

Analgesic activity in mice after s.c. injection of some highly μ-selective analogs in the PBQ writhing test is shown in Table II. Analogs I, V and VI showed low analgesic activity, similar to that of the lead peptide. Penetration to the brain across the blood brain barrier (BBB) of I, V and VI having two positive charges in the molecule after peripheral administration remains to be investigated in the future. Based on the assumption that highly polar, especially basic, peptide derivatives would not cross the BBB, analgesic compounds which act peripherally are being developed.[11,12] And it is advocated that one of these compounds, Tyr–D-Arg–Phe–Lys–NH_2, having three positive charges, is a peripherally active analgesic.[11] On the contrary, based on the assumption that there is a system of anionic receptor or binding sites in BBB-mediated transcytosis,[13] MeTyr–Gly–Gly–Phe–Leu–Arg–MeArg–D-Leu–NHC_2H_5, having three positive charges, has been shown to cross the BBB.[14] The apparent discrepancies need to be investigated further. For the solution of these problemes, I, V and VI may be good reagents.

In conclusion, the present study demonstrates that Tyr–D-Arg–Phe–βAla–NH_2 (I) is potent and one of the most selective μ-receptor agonists reported to date, and the fluorinated analogs of the Tyr^1 aromatic ring at position 2 or 3 retains potent μ-receptor affinity and high selectivity similar to I. Since I, V and VI resist enzymatic degradation, it is expected that these peptides are excellent reagents for studying the function of μ-receptor-mediated biological properties *in vitro* and *in vivo*.

TABLE II. PBQ-Induced Writhing Test of Highly μ-Selective Analogs

Peptide	ED_{50}[a] (μg/kg, s.c.)
Tyr–D-Arg–Phe–βAla	24.9 (18.3—33.9)
I	56.6 (46.2—69.3)
V	134 (107—168)
VI	140 (112—174)

a) The 95% confidence limits are given in parentheses.

Experimental

Optical rotations were measured with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel plates (Merck, Kieselgel $60F_{254}$, 5 × 10 cm) with the following solvent systems: *Rf*(A), 1-BuOH–AcOH–H_2O (4:1:5, upper phase); *Rf*(B), 1-BuOH–AcOH–pyridine–H_2O (15:3:10:12). Analytical HPLC was performed on a YMC-Pack AM-303 ODS column (4.6 × 250 mm) by gradient elution with the following solvent system: A, 0.06% trifluoroacetic acid (TFA) in H_2O and B, 80% acetonitrile in 0.06% TFA. A linear gradient from 10% B to 50% B over 40 min at a flow rate of 1.2 ml/min was used, and the eluate was monitored at 215 nm. Amino acid analysis was carried out on a Hitachi 835 analyzer using a high separation column after 6 N HCl hydrolysis of the peptide at 110 °C for 20 h. Fluorinated amino acids were a generous gift from Asahi Glass Co., Ltd.

Solid Phase Peptide Synthesis The peptide was constructed on Boc–βAla–benzhydrylamine resin (0.85 meq/g, 1% cross-linked, 100—200 mesh) according to the coupling schedule described previously.[3] Boc amino acids with side chain protecting groups, 2-bromobenzyloxycarbonyl for Tyr and tosyl for D-Arg, were used. The hydroxyl group of fluorinated Tyr was left unprotected. The protected peptide resin was treated with anhydrous HF containing 10% anisole at 0 °C for 60 min. After evaporation of excess HF under vacuum, the resulting residue was extracted with 5% AcOH. The extract was washed with ether and evaporated to dryness under vacuum. The crude peptide was purified on a Develosil LOP ODS 24S (Nomura Kagaku) column (24 × 360 cm) which was eluted with a linear gradient of 12—32% acetonitrile in 0.1% TFA over 150 min at a flow rate of 3 ml/min. The eluate was monitored at 280 nm. Fractions around the main peak were checked by analytical HPLC and the pure parts were collected and freeze-dried. The synthetic peptide was converted to its AcOH salt by treatment with Dowex 1 × 2 (AcOH form) resin. Analytical data of peptides synthesized are shown in Table III.

Opioid Receptor Binding Assay The binding assay was performed by the method previously described in detail.[1a] [^{3}H]DAGO, [^{3}H]DPDPE and [^{3}H]U-69593 were used as radioligands for μ, δ and κ-receptors, respectively. Inhibition constants (K_i) were calculated from IC_{50} values using the relation of $K_i = IC_{50}/(1 + L/K_d)$,[15] where L is a concentration of a 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, [^{3}H]DPDPE and [^{3}H]U-69593 used are 0.46, 3.43 and 0.62, respectively.

TABLE III. Analytical Data of Synthetic Peptides

Compound	$[\alpha]_D$[a] (°)	TLC[b]		Amino acid analysis				HPLC[b]
		Rf(A)	*Rf*(B)	Tyr[c]	D-Arg	Phe[c]	βAla[d]	t_R (min)
I	+29.8	0.33	0.68	0.97	1.14	1.00	0.95	19.0
II	+30.7	0.37	0.75	0.68	1.00	0.55[c]	1.00	20.0
III	+35.1	0.36	0.74	0.88	1.00	+[e]	+[e]	20.9
IV	+28.3	0.36	0.76	0.92	1.00	0.89[c]	1.12	20.9
V	+29.3	0.38	0.74	+[f]	1.00	0.93	+[f]	20.0
VI	+29.1	0.35	0.74	0.92[c]	1.00	0.98	1.00	19.9
VII	+33.9	0.41	0.74	+[f]	1.00	0.87	+[f]	22.0

a) Optical rotation was measured in 1% AcOH (c = 0.5) at 20 °C. *b*) See Experimental. *c*) See ref. 3 for retention times of fluorinated Tyr and Phe on amino acid analyzer. *d*) βAla was eluted just before Phe. *e*) Phe(3F) + βAla = 1.19 as Phe. *f*) Tyr(3F) + βAla = 2.20 as Phe.

Enzymatic Stability of Peptides in a Rat Brain Homogenate A peptide (100 μg) was incubated with 400 μl of crude rat synaptosomal membrane fractions[1a] (0.73 mg protein/ml) in 50 mM Tris–HCl buffer at pH 7.40 at 36 °C for 5 h. The reaction was stopped by addition of 0.2 M HCl (50 μl). After centrifugation at 10000 r.p.m. for 10 min, an aliquot of the supernatant was applied to analytical HPLC. No degradation products were detected on HPLC in any sample of peptides synthesized in this study. Under the incubation conditions, Met-enkephalin was degraded completely within 40 min.

PBQ Writhing Test Groups of six male mice of the ICR Strain weighing 15—18 g were used. Peptides dissolved in saline solution or saline alone were administered subcutaneously in a dose volume of 10 ml/kg, and 15 min later the mice were injected with PBQ (2.5 mg/kg) intraperitoneally. Five minutes after the PBQ injection, abdominal torsion and contraction was counted for 10 min. ED_{50} values were defined as the dose of peptides capable of reducing writhing in saline-injected mice by 50%. ED_{50} and 95% confidence limits were determined by the method of Litchfield and Wilcoxon.[16]

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Agonistic and Antagonistic Activities of Neuromedin U-8 Analogs Substituted with Glycine or D-Amino Acid on Contractile Activity of Chicken Crop Smooth Muscle Preparations[1)]

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To study the structure–activity relationships of neuromedin U-8 (NMU-8) (H–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2) and to develop a NMU-8 antagonist, twenty-three NMU-8 analogs substituted with Gly or the corresponding D-amino acid(s) at positions 1—8 were synthesized by solid-phase techniques. On isolated chicken crop preparations, the contractile activity of the synthetic NMU-8 analogs was compared with that of NMU-8 and their antagonistic activity was assayed against NMU-8. The replacement of Phe^2, Phe^4, Arg^5, Pro^6, Arg^7 or Asn^8 with Gly brought about a drastic decrease of the agonistic activities. Substitution of the corresponding D-amino acid residue for Phe^2, Phe^4, Arg^5, Pro^6 or Asn^8 caused a marked decrease of the agonistic activities, while the replacement of Tyr^1 with D-form enhanced the activity. It was further revealed that [D-Pro^6]–NMU-8 and [D-Leu^3, D-Pro^6]–NMU-8 exerted a non-competitive antagonistic activity against NMU-8 with x values of 5.22 ± 0.12 and 5.34 ± 0.09, respectively. [D-Phe^2, D-Pro^6]–NMU-8, [D-Arg^5, D-Pro^6]–NMU-8 and [D-Pro^6, D-Asn^8]–NMU-8 showed a very weak antagonism. The results indicated that 1) the side chain of each amino acid at positions 2, 4, 5, 6, 7 and 8 of NMU-8 is of relative importance for the expression of the contractile activity, and 2) [D-Pro^6]–NMU-8 and its four analogs acted as an antagonist against NMU-8.

Keywords neuromedin U-8; glycine analog; D-amino acid analog; solid-phase synthesis; structure–activity relationships; chicken crop; smooth muscle contraction; contractile activity; antagonistic activity; antagonist

In 1985, Minamino *et al.*[2)] elucidated the primary structure of novel neuropeptides, neuromedin U-25 (NMU-25) and U-8 (NMU-8) (Fig. 1), isolated from porcine spinal cord. NMU-25 contains the NMU-8 sequence at its C-terminus, preceded by paired Arg residues. Recently, rat neuromedin U (r-NMU),[3,4)] frog neuromedin U (f-NMU),[5)] human neuromedin U (h-NMU)[6)] and guinea pig neuromedin U[7)] were isolated, and their structures (Fig. 1) were determined. A common C-terminal sequence, Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2, in the mammlian neuromedin Us and the sequence similarity of the N-terminal region of NMU-25, r-NMU, h-NMU and f-NMU have been proved. Neuromedin Us have a potent contractile effect on isolated rat uterus[2)] and chicken crop[8)] smooth muscle preparations, cause an increase in blood pressure in rats,[2)] and exert the effects on splanchnic blood flow in dogs.[9)] NMU-25 is approximately three times more potent than NMU-8 in contracting isolated rat uterus preparation.[2)] These results indicate that the NMU-8 sequence may be essential for exerting stimulant effect on rat uterus preparations, and that the N-terminal part of NMU-25 may serve to reinforce the activity.[2)] The peptide chain length–contractile activity relationships[2,10)] of NMU-8 on isolated smooth muscle preparations and a role[2)] in biological activity of α-amide at the C-termimus have been reported. No NMU-8 or NMU antagonist has been reported in the literature.

To study the relationship between the structure and function of NMU-8 and to develop a specific and competitive antagonist against NMU-8, we used a systematic approach to examine two series of Gly or D-amino acid substituted NMU-8 analogs (Fig. 2). In the first series, each amino acid residue of NMU-8 was replaced singly by Gly residue to examine the role of the side chain in the contractile activity on isolated smooth muscle preparations and to develop a NMU-8 antagonist. Our earlier study[11)] on the structure–activity relationship of neurokinin B (H–Asp–Met–His–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2) revealed that the replacement of Phe^5 residue with Gly moiety changes the pharmacological spectrum of neurokinin B from that of an agonist to that of an antagonist.[12)] We have succeeded in the development of several specific antagonists against neurokinin B.[13)] Therefore, Gly substitution seems a useful tool for the development of a NMU-8 antagonist. In the second series, each amino acid was replaced by its corresponding D-amino acid, primarily to examine an antagonistic activity against NMU-8. The biological activities of the synthetic peptides were examined on isolated chicken crop smooth muscle preparation.[8)]

Methods

Synthesis Synthesis of the NMU-8 analogs used in this study was performed on a Beckman system 990C peptide synthesizer by standard solid phase method[14)] starting from Boc-amino acid benzhydrylamine resin (1% divinylbenzene copolymer; Peptide Institute Inc. Japan: amino acid content; 0.3 mmol/g of resin). Boc protection was used for α-amino function. The side-chain protective groups used were *p*-tosyl for the guanidino group of Arg and 2,6-dichlorobenzyl for the hydroxyl group of Tyr. Elongation of peptide chain using 2.5 fold equivalent of Boc-amino

		Sequence (positions 1, 5, 10, 15, 20, 25)
porcine	neuromedin U–25	H–Phe–Lys–Val–Asp–Glu–Glu–Phe–Gln–Gly–Pro–Ile–Val–Ser–Gln–Asn–Arg–Arg–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
porcine	neuromedin U–8	H–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
guinea pig	neuromedin U	H–Gly–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
rat	neuromedin U	H–Tyr–Lys–Val–Asn–Glu– –Tyr–Gln–Gly–Pro– –Val–Ala–Pro–Ser–Gly–Gly–Phe–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
human	neuromedin U	H–Phe–Arg–Val–Asp–Glu–Glu–Phe–Gln–Ser–Pro–Phe–Ala–Ser–Gln–Ser–Arg–Gly–Tyr–Phe–Leu–Phe–Arg–Pro–Arg–Asn–NH_2
frog	neuromedin U	H–Leu–Lys–Pro–Asp–Glu–Glu–Leu–Gln–Gly–Pro–Gly–Gly–Val–Leu–Ser–Arg–Gly–Tyr–Phe–Val–Phe–Arg–Pro–Arg–Asn–NH_2

Fig. 1. Amino Acid Sequences of Neuromedin Us

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	Analog		1	2	3	4	5	6	7	8
1	[Gly^{1}]-NMU-8	H	Gly	Phe	Leu	Phe	Arg	Pro	Arg	Asn-NH_2
2	[Gly^{2}]-NMU-8	H	Tyr	Gly	Leu	Phe	Arg	Pro	Arg	Asn-NH_2
3	[Gly^{3}]-NMU-8	H	Tyr	Phe	Gly	Phe	Arg	Pro	Arg	Asn-NH_2
4	[Gly^{4}]-NMU-8	H	Tyr	Phe	Leu	Gly	Arg	Pro	Arg	Asn-NH_2
5	[Gly^{5}]-NMU-8	H	Tyr	Phe	Leu	Phe	Gly	Pro	Arg	Asn-NH_2
6	[Gly^{6}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	Gly	Arg	Asn-NH_2
7	[Gly^{7}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	Pro	Gly	Asn-NH_2
8	[Gly^{8}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	Pro	Arg	Gly-NH_2
9	[D-Tyr^{1}]-NMU-8	H	D-Tyr	Phe	Leu	Phe	Arg	Pro	Arg	Asn-NH_2
10	[D-Phe^{2}]-NMU-8	H	Tyr	D-Phe	Leu	Phe	Arg	Pro	Arg	Asn-NH_2
11	[D-Leu^{3}]-NMU-8	H	Tyr	Phe	D-Leu	Phe	Arg	Pro	Arg	Asn-NH_2
12	[D-Phe^{4}]-NMU-8	H	Tyr	Phe	Leu	D-Phe	Arg	Pro	Arg	Asn-NH_2
13	[D-Arg^{5}]-NMU-8	H	Tyr	Phe	Leu	Phe	D-Arg	Pro	Arg	Asn-NH_2
14	[D-Pro^{6}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	D-Pro	Arg	Asn-NH_2
15	[D-Arg^{7}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	Pro	D-Arg	Asn-NH_2
16	[D-Asn^{8}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	Pro	Arg	D-Asn-NH_2
17	[D-Tyr^{1}, D-Pro^{6}]-NMU-8	H	D-Tyr	Phe	Leu	Phe	Arg	D-Pro	Arg	Asn-NH_2
18	[D-Phe^{2}, D-Pro^{6}]-NMU-8	H	Tyr	D-Phe	Leu	Phe	Arg	D-Pro	Arg	Asn-NH_2
19	[D-Leu^{3}, D-Pro^{6}]-NMU-8	H	Tyr	Phe	D-Leu	Phe	Arg	D-Pro	Arg	Asn-NH_2
20	[D-Phe^{4}, D-Pro^{6}]-NMU-8	H	Tyr	Phe	Leu	D-Phe	Arg	D-Pro	Arg	Asn-NH_2
21	[D-Arg^{5}, D-Pro^{6}]-NMU-8	H	Tyr	Phe	Leu	Phe	D-Arg	D-Pro	Arg	Asn-NH_2
22	[D-Pro^{6}, D-Arg^{7}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	D-Pro	D-Arg	Asn-NH_2
23	[D-Pro^{6}, D-Asn^{8}]-NMU-8	H	Tyr	Phe	Leu	Phe	Arg	D-Pro	Arg	D-Asn-NH_2

Fig. 2. Amino Acid Sequences of Synthetic NMU-8 Analogs

TABLE I. Amino Acid Analyses of the Synthetic NMU-8 Analogs

Analog	Asp	Pro	Gly	Leu	Tyr	Phe	Arg	NH_3
1	1.03 (1)	0.92 (1)	1.04 (1)	1.04 (1)	—	2.02 (2)	1.95 (2)	2.46 (2)
2	1.04 (1)	0.97 (1)	1.02 (1)	1.01 (1)	0.99 (1)	1.02 (1)	1.95 (2)	2.32 (2)
3	1.04 (1)	0.92 (1)	1.00 (1)	—	1.01 (1)	2.06 (2)	1.97 (2)	2.05 (2)
4	1.02 (1)	0.92 (1)	1.02 (1)	1.03 (1)	0.98 (1)	1.02 (1)	2.01 (2)	2.43 (2)
5	1.04 (1)	0.91 (1)	1.02 (1)	1.04 (1)	1.02 (1)	2.07 (2)	0.92 (1)	2.26 (2)
6	1.01 (1)	—	0.99 (1)	1.01 (1)	0.97 (1)	1.98 (2)	2.04 (2)	2.33 (2)
7	1.02 (1)	0.91 (1)	1.01 (1)	1.04 (1)	1.02 (1)	2.07 (2)	0.93 (1)	2.15 (2)
8	—	0.96 (1)	1.00 (1)	1.03 (1)	1.00 (1)	2.04 (2)	1.98 (2)	1.39 (1)
9	1.02 (1)	0.99 (1)	—	1.01 (1)	0.97 (1)	1.99 (2)	2.03 (2)	2.45 (2)
10	1.03 (1)	0.97 (1)	—	1.01 (1)	0.97 (1)	1.98 (2)	2.04 (2)	2.43 (2)
11	1.03 (1)	0.99 (1)	—	1.00 (1)	0.96 (1)	1.97 (2)	2.05 (2)	2.64 (2)
12	1.04 (1)	0.98 (1)	—	1.00 (1)	0.97 (1)	1.96 (2)	2.05 (2)	2.24 (2)
13	1.06 (1)	0.97 (1)	—	1.01 (1)	0.97 (1)	1.97 (2)	2.01 (2)	2.51 (2)
14	1.04 (1)	0.99 (1)	—	1.00 (1)	0.97 (1)	1.96 (2)	2.03 (2)	2.63 (2)
15	1.02 (1)	0.97 (1)	—	1.00 (1)	0.97 (1)	1.97 (2)	2.08 (2)	2.38 (2)
16	1.03 (1)	0.97 (1)	—	0.99 (1)	0.97 (1)	1.96 (2)	2.08 (2)	2.13 (2)
17	1.03 (1)	1.01 (1)	—	1.00 (1)	0.97 (1)	2.00 (2)	2.02 (2)	2.41 (2)
18	1.03 (1)	0.97 (1)	—	1.08 (1)	0.93 (1)	1.99 (2)	2.00 (2)	2.64 (2)
19	1.03 (1)	0.96 (1)	—	1.04 (1)	1.00 (1)	1.98 (2)	1.99 (2)	2.53 (2)
20	1.01 (1)	0.99 (1)	—	1.02 (1)	0.98 (1)	1.98 (2)	2.03 (2)	2.00 (2)
21	1.02 (1)	1.00 (1)	—	1.02 (1)	0.98 (1)	1.97 (2)	2.01 (2)	2.03 (2)
22	1.02 (1)	1.00 (1)	—	1.02 (1)	0.98 (1)	1.98 (2)	2.00 (2)	2.07 (2)
23	1.01 (1)	0.99 (1)	—	1.02 (1)	0.98 (1)	1.98 (2)	2.02 (2)	2.01 (2)

acids was carried out with DCC in CH_2Cl_2 and/or DMF. A 2-fold excess of HOBt was used for coupling reaction of Asn. Deprotection of N^{α}-amino group during the peptide chain elongations was executed by 33% TFA in CH_2Cl_2 for 30 min. The quantification of coupling reaction was carried out by a negative ninhydrin color test. Unreacted amino groups were blocked by acetylation using ten fold of acetic anhydride and pyridine in CH_2Cl_2 for 20 min. After completion of the chain elongation, the protected peptide resin was simultaneously cleaved and deblocked with anhydrous liquid HF[15] containing 10% anisole. The reaction mixture was kept at −20 °C for 30 min and then 0 °C for 30 min. After evaporation of HF *in vacuo* with ice-cooling, the peptide resin mixture was washed with AcOEt prior to extraction of the peptide with diluted AcOH. The extracts

combined were lyophilized. The crude product was purified by preparative RP-HPLC composed of a Waters model 590 pump and a Waters U6K injector connected to a column (19×150 mm) of μ-Bondasphere C_{18} 5-100A. The eluates were monitored with a Soma UV detector S-310A model-II at 210 nm wavelength. MeCN–0.1% TFA solvent system was used as eluent at a flow rate of 10 ml min^{-1}. Each peptide emerged at 40—60 min by isocratical elution with 14—20% MeCN contents of the solvent system. The desired fraction was desalted on a Sephadex G-25 (super fine) eluted with 12% AcOH and the product was lyophilized. Homogeneity of the peptides was ascertained by analytical HPLC on Nova-pak C_{18} (3.9×150 mm) eluted with 20 mM phosphate buffer (pH 3.0)–MeCN, HP-TLC (Merck) with solvent systems of *n*-BuOH–AcOH–H_2O (4:1:5, Rf^1) and *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, Rf^2). Hydrolysis of peptides was carried out at 110 °C for 24 h in 6 N HCl containing phenol. Amino acid analysis (Table I) of the acid hydrolysate was performed by a Beckmann model 7300 amino acid analyzer system. When single peaks and single spots were observed for a peptide in all chromatographic systems and amino acid composition after hydrolysis of the peptides was consistent with the calculated values, the peptides were submitted for bioassay. Characterization of the synthetic peptides is shown in Table II.

Bioassay Biological properties of NMU-8 analogs were examined in isolated chicken crop preparations. Chickens (age up to 10 d) were killed by bleeding from the neck. The desired organ was taken out rapidly. The chicken crop was cut vertically (about 1×30 mm). The preparation was suspended in an organ bath (10 ml) containing Tyrode's solution gassed with a mixture of 95% O_2 and 5% CO_2 and kept at 28 °C. The muscle preparations were equilibrated for 60 min under a load of 0.5 g and washed every 20 min. The bathing fluid was of the following composition: NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.1 mM, NaH_2PO_4 0.4 mM, $NaHCO_3$ 12 mM, glucose 5.6 mM.

Concentration–response curves of NMU-8 and its analogs were obtained cumulatively in order to estimate the apparent affinities as agonists. Responses were recorded using an isotonic transducer (Nihon Kohden, TD-111T) on a recorder (Hitachi, 561—3003). All the peptides were applied up to a concentration of 10^{-5} M to test their contractile activities. The antagonistic effects of the NMU-8 analogs were assessed by analysis of the cumulative dose–response curves at three concentrations (2×10^{-6}, 5×10^{-6} and 10^{-5} M) of the NMU-8 analogs in the bath [added 10 min before an application of NMU-8]. The pharmacological parameter of relative affinities (RA) (EC_{50} of NMU-8/EC_{50} of each stimulant) was used for the characterization as agonists of the NMU-8 analogs. *x* [empirical *x*-intercept (pA_2 in the case of a competitive antagonist) of the line of Schild plot[16] as determined by the linear regression analysis in the case of complex antagonism] and the slope value of the Schild plot were determined for characterization of the antagonism of NMU-8 analogs against NMU-8.

Results and Discussion

The biological activities of the synthetic peptides were examined on isolated chicken crop smooth muscle preparation.[8] The contractile activities of the NMU-8 analogs were compared with that of synthetic NMU-8. The results are shown in Table III. The substitution of Gly moiety for each amino acid residue at positions 2, 4, 5, 6, 7 and 8 of NMU-8 brought about drastic decrease of the intrinsic activity. Analogs **2**, **4**, **5**, **6** and **8** possessd a little activity and analog **7** showed none, while analogs **1** and **3** had 40% and 8% of the activity of NMU-8, respectively. Thus, it is suggested that the side chains of each amino acid residue at positions 2—8 of NMU-8 are of relative importance for the expression of the contractile activity on isolated chicken crop smooth muscle. All the Gly substituted analogs were tested for the ability to antagonize the myotropic actions of NMU-8, and failed to antagonize.

The substitution of D-amino acid moiety for each amino acid residue of NMU-8 is the next reasonable approach in

Table II. Physical Properties and Yield of Synthetic NMU-8 Analogs

Analog	$[\alpha]_D^{23}$ (°) (c=0.5, 2 M AcOH)	RP-HPLC[a] t_R[c] (min)	HP-TLC[b] Rf^1	Rf^2	Yield (%)
1	−47.6	4.27	0.02	0.50	15.8
2	−42.4	2.63	0.03	0.50	33.4
3	−28.4	3.38	0.02	0.49	32.1
4	−40.4	2.15	0.02	0.48	28.5
5	−30.8	11.69	0.12	0.60	34.0
6	−22.8	5.99	0.05	0.53	27.7
7	−32.8	10.32	0.12	0.60	48.5
8	−40.0	8.02	0.06	0.56	53.3
9	−55.6	8.99	0.05	0.55	29.9
10	−37.6	13.36	0.07	0.56	35.7
11	−18.8	12.59	0.06	0.53	22.2
12	−37.6	10.11	0.06	0.54	30.5
13	−44.0	6.36	0.04	0.53	33.4
14	−15.6	7.58	0.05	0.53	14.9
15	−23.2	7.90	0.05	0.54	46.1
16	−36.8	7.90	0.05	0.54	32.5
17	−23.9	9.45	0.03	0.51	40.1
18	−18.7	14.37	0.02	0.50	44.0
19	+15.4	12.67	0.02	0.49	22.9
20	+ 9.6	11.35	0.01	0.57	47.1
21	− 9.6	8.28	0.00	0.59	53.8
22	+ 4.4	6.43	0.00	0.55	33.5
23	− 2.8	8.28	0.00	0.56	25.4

a) Column, Nova-pak C_{18}; flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 17.5% to 35% MeCN (15 min) in 20 mM phosphate buffer (pH 3.0). *b*) Rf^1, *n*-BuOH–AcOH–H_2O (4:1:5); Rf^2, *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24). *c*) Retention time.

Table III. Contractile Activity of Synthetic NMU-8 Analogs on Isolated Chicken Crop Smooth Muscle Preparations

Analog	RA[a]	Analog	RA	Analog	RA
1	0.40±0.12	**9**	2.47±0.17	**17**	0.09±0.03
2	<0.01	**10**	<0.09	**18**	NA
3	0.08±0.02	**11**	0.33±0.02	**19**	0.04±0.01
4	<0.01	**12**	<0.08	**20**	0.13±0.04
5	<0.01	**13**	<0.11	**21**	0.12±0.04
6	<0.01	**14**	—	**22**	—
7	—	**15**	0.75±0.18	**23**	NA
8	<0.05	**16**	<0.12		

a) Relative affinity (EC_{50} of NMU-8/EC_{50} of each NMU-8 analog) to NMU-8. —: no contractile effect. NA: not assessed. Each value is the average±S.E.M. of 6—11 determinations.

Table IV. Antagonistic Activity of Synthetic NMU-8 Analogs on Isolated Chicken Crop Smooth Muscle Preparations

Analog	x[a]	Slope[b]	n[c]
10	—	—	6
12	—	—	6
13	—	—	6
14	5.22±0.12	−1.52±0.14	6
16	—	—	6
17	—	—	6
18	<5	NA	6
19	5.34±0.09	−2.25±0.32	6
20	—	—	5
21	<5	NA	8
22	—	—	6
23	<5	NA	6

a) Empirical *x*-intercept (pA_2 in the case of a competitive antagonist) of the line of Schild plot as determined by linear regression analysis. *b*) Slope of the line of Schild plot as determined by linear regression analysis. *c*) Number of experiments. —: the analog failed to antagonize the response to NMU-8. NA: not assessed.

the search for a NMU-8 antagonist: replacement of natural amino acid in a bioactive peptide with a D-moiety is a useful tool for reducing agonistic activity or for changing the activity from agonistic to antagonistic. Substitution of the corresponding D-moiety for Phe^2, Phe^4, Arg^5, or Asn^8 gave rise to analogs with a marked decrease of agonistic activities, and **14** showed no contractile effect. The potencies of **11** and **15** were 33% and 75% of NMU-8, respectively. The replacement of Tyr^1 with D-form enhanced the activity, presumably by resistance to aminopeptidase-like proteolytic degradation. The potency of **9** was twice as high as that of NMU-8 itself. Then, analogs **10**, **12**, **13**, **14** and **16**, which showed low or no contractile activity, were tested for the antagonistic activity against NMU-8. Analogs **10**, **12**, **13** and **16** were inactive as an antagonist, while [D-Pro^6]–NMU-8 **14** acted as an antagonist against NMU-8 (Table IV). Analog **14** produced a concentration-dependent rightward shift of the curve to NMU-8. The x value was 5.22 ± 0.12. A Schild plot calculated from the results of experiments with three concentrations of **14** gave a straight line the slope of which was significantly different from unity, indicating complex antagonism. The slope value was -1.52 ± 0.14. The results indicated that the replacement of an important amino acid residue for intrinsic activity by D-form is beneficial for the development of an antagonist against NMU-8. In this case D-Pro substitution appears to stabilize the conformation of the analog that binds to the receptor, but does not provoke receptor stimulation.

Analogs **17**—**23** were designed by replacement of the natural amino acid residues of [D-Pro^6]–NMU-8 **14** with the corresponding D-amino acid, to develop a more potent antagonist against NMU-8. The seven analogs possessed weak or no agonistic activities (Table III). Therefore, all analogs were tested for antagonistic effect against NMU-8 (Table IV). The substitution of D-form for position 1, 4, or 7 at **14** led to the loss of the antagonistic activity of **14** against NMU-8. Analogs **17**, **20** and **22** failed to antagonize the response to NMU-8. The incorporation of D-amino acid moiety into position 2, 5, or 8 did not result in an enhancement of the antagonistic activity of **14**. Analogs **18**, **21** and **23** acted as very weak antagonists against NMU-8; because the x values of the three analogs were less than 5, the slope values were not assessed. Analog **19** retained antagonistic activity at the same magnitude as **14**: the antagonist caused a shift of the concentration–response curve for NMU-8 to the right. The x value was 5.34 ± 0.09. Slope of the Schild plot calculated from the results of experiments with three concentrations of **19** was significantly different from unity, suggesting a non-competitive antagonism. The slope value was -2.25 ± 0.32. Thus, the introduction of the corresponding D-form into position 1, 2, 3, 4, 5, 7, or 8 of [D-Pro^6]–NMU-8 **14** was unfavorable for a more potent and competitive antagonism.

In the present study, the results indicated that the side chain of each amino acid at positions 2—8 of NMU-8 is of relative importance for the expression of contractile activity in isolated chicken crop smooth muscle preparations. Furthermore, it was revealed that four NMU-8 analogs exerted an antagonistic activity against NMU-8. The antagonistic activity of [D-Pro^6]–NMU-8 was as high as that of [D-Leu^3, D-Pro^6]–NMU-8. However, both antagonists showed non-competitive antagonism. The others acted as poorer antagonists against NMU-8. The finding that the replacement of Pro moiety at position 6 on NMU-8 with D-form changed the pharmacological spectrum of NMU-8 from that of an agonist to that of an antagonist is an important clue in the development of NMU-8 antagonist. These results could help in the design of a more potent and specific NMU-8 antagonist.

References and Notes

1) Amino acids and their derivatives except glycine mentioned in this paper are of L-configuration unless otherwise indicated. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbriviations used are: Boc, *tert*-butoxycarbonyl; Tos, *p*-toluenesulfonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, *N,N*-dimethylformamide; MeCN, acetonitrile; AcOH, acetic acid; TFA, trifluoroacetic acid; BuOH, butyl alcohol; AcOEt ethyl acetate; RP-HPLC, reverse phase high performance liquid chromatography; HP-TLC, high performance thin layer chromatography.
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Preparation and Biological Activity of 2-[4-(Thiazol-2-yl)phenyl]propionic Acid Derivatives Inhibiting Cyclooxygenase

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A series of 2-[4-(thiazol-2-yl)phenyl]propionic acids substituted at various positions were prepared by the reaction of diethyl 2-methyl-2-(4-thiocarbamoylphenyl)malonates with α-bromoaldehyde diethyl acetals or α-haloketones followed by hydrolysis of esters. The inhibition of prostaglandin H synthetase (cyclooxygenase) was assayed by use of an enzyme preparation from guinea pig polymorphonuclear leukocytes. Examination of the structure–activity relationship of these compounds indicated that the substitution pattern with halogens at position 3 (R_1) of the benzene ring and a methyl group in position 4 (R_2) and/or 5 (R_3) of the thiazole ring were favorable for inhibitory activity. The compounds bearing bulky alkyl or polar functional groups at the R_2 position were weak inhibitors. The potent inhibitors of cyclooxygenase were tested for their ability to reduce carrageenin-induced inflammation of rat paws. These derivatives had strong anti-inflammatory activity based on their strong inhibition of cyclooxygenase, with some exceptions, including those with a thiomethyl group at R_1.

Keywords structure–activity relationship; cyclooxygenase inhibition; anti-inflammatory; carrageenin-induced inflammation; thiazole derivative; phenylpropionic acid

The anti-inflammatory activity of non-steroidal anti-inflammatory compounds is related to their ability to inhibit cyclooxygenase.[1] The relationship between the inhibition of carrageenin-induced edema and that of prostaglandin biosynthesis *in vivo* (the reduction of urinary prostaglandin E_2 excretion) has been clearly defined.[2] In addition, the relationship between inhibitions of the edema induction and prostaglandin biosynthesis *in vitro* with cyclooxygenase preparations has been explained not directly but in conjunction with such physicochemical parameters of the molecule as hydrophobicity in terms of log P, P being the 1-octanol/water partition coefficient.[3] The mechanism of cyclooxygenase inhibition has been extensively studied by the use of a purified preparation of prostaglandin H synthetase.[4]

R_1 = H, F, Cl, Br, CF_3, Me, OMe, SMe, OH, NO_2, NH_2

R_2 = H, Me, Et, iso-Pr, Bu, acetyl, Ph, vinyl, allyl, CH_2OH, CH_2OMe, $CONH_2$, CONHMe, $CONMe_2$, CF_3

R_3 = H, Me, Et $\quad R_2, R_3 = -(CH_2)_n-$; $n = 3, 4$

A number of 2-arylpropionic acids such as ibuprofen[5] and flurbiprofen[6] are anti-inflammatory agents. Some analogs containing the thiazole ring in the aryl moiety or as a part of the substituents are anti-inflammatory.[7] Being interested in further exploration of anti-inflammatory arylpropionic acids with thiazole rings, we synthesized a number of substituted 2-[4-(thiazol-2-yl)phenyl]propionic acids. We found that these compounds inhibit cyclooxygenase to various extents depending upon the substitution. In this report, we describe the synthesis of these compounds and the relationship between their structure and cyclooxygenase inhibition. Some compounds that strongly inhibited this enzyme also had strong anti-inflammatory activity in suppressing carrageenin-induced edema in rats.

	X_1	R_1
I-1	Cl	H
I-2	F	F
I-3	Cl	Cl
I-4	Cl	Br
I-5	Cl	CF_3
I-6	F	Me
I-7	F	OMe

I → (NaH, $MeCH(CO_2Et)_2$) → II → (1) H_2 Pd/C 2) $NaNO_2$, $KCu(CN)_2$) → III → (H_2S) → IV → ($CH_2BrCH(OEt)_2$) → V → (KOH) → 1–8

R_1 = H, F, Cl, Br, CF_3, Me, OCH_3, SMe

Chart 1

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Chemistry

The compounds were synthesized by Hantzsch thiazole synthesis.[8] The 2-[3-substituted-4-(thiazol-2-yl)phenyl]propionic acids **1**—**8** were prepared as shown in Chart 1. The diethyl 2-methyl-2-(3-substituted-4-nitrophenyl)malonates, II-1, II-3, II-4, and II-5, were obtained by the reaction of the corresponding 4-chloro-2-substituted-nitrobenzenes, I-1, I-3, I-4, and I-5, with diethyl methyl malonate. The malonates, II-2, II-6, and II-7, were also obtained by the reaction of 4-fluoro-2-substituted-nitrobenzenes, I-2, I-6, and I-7. 4-Chloro-2-bromonitrobenzene, I-4, was derived from 4-chloro-2-aminonitrobenzene by use of the Sandmeyer reaction.[9] 4-Fluoro-2-methoxynitrobenzene, I-7, were derived from 4-fluoro-2-hydroxynitrobenzene by use of methylation. Diethyl 2-methyl-2-(3-methylthio-4-nitrophenyl)malonate (II-8), was obtained by the reaction of diethyl 2-(3-chloro-4-nitrophenyl)-2-methylmalonate (II-3), with methanethiol derived from dimethyldisulfide and tri-*n*-butylphosphine.

Catalytic hydrogenation of compounds II gave amino derivatives that were converted by use of the Sandmeyer reaction to cyano derivatives III, which were easily converted to thioamide derivatives IV. The thioamides were converted to thiazole derivatives V by use of Hantzsch thiazole synthesis followed by hydrolysis to give compounds **1**—**8**. 2-[3-Hydroxy-4-(thiazol-2-yl)phenyl]propionic acid (**9**), was derived from the demethylation[10] of the corresponding methoxy derivative **7**. For the synthesis of **10**, we used another method (Chart 2), because methyl 4-cyano-3-nitrobenzoate was not obtained by the Sandmeyer reaction of methyl 4-amino-3-nitrobenzoate. Methyl 4-carbamoyl-3-nitrobenzoate (VII), was prepared by selective esterification of nitroterephthalic acid followed by amidation. The carbamoyl group obtained was converted to a thiazole group by reaction with phosphorous pentasulfide followed by Hantzsch thiazole synthesis. The benzoate VIII obtained was converted to compound **10** by Wolf rearrangement[11] followed by hydrolysis. The amino derivative **11** was prepared by the reduction of the nitro group of IX followed by hydrolysis.

Derivatives bearing various substituents at R_1, R_2, and R_3 (**12**—**17**, **28**—**35**, **38**—**43**, and **46**—**47**) were also synthesized by Hantzsch thiazole synthesis[8] (Chart 3). 1-Chloro-3-methylbutan-2-one and 1-chlorohexan-2-one were prepared by the diazomethane-mediated chloromethylation[12] of the corresponding carboxylic acid chlorides. 2-Bromopentan-3-one and 2-bromocyclohexanone were prepared by the bromination[13] of the correspond-

VI → 1) MeOH H^+ 2) $SOCl_2$ NH_4OH → VII → 1) P_2S_5 2) $CH_2BrCH(OEt)_2$ → VIII → 1) NaOH 2) $SOCl_2$ CH_3CHN_2 $PhCH_2OH$ → IX : $R_1 = NO_2$; X : $R_1 = NH_2$ → KOH → **10** : $R_1 = NO_2$; **11** : $R_1 = NH_2$

Chart 2

IV → $R_2COCH(X_2)R_3$, X_2 = Cl, Br → XI → NaOH → **12-17, 28-35, 38-43, 46-47**

R_1=H, Cl, F; R_2=Me, Et, iso-Pr, Bu, acetyl, phenyl ; R_3=H, Me, Et

Chart 3

XII → IV-1 → XIII → NaOH → **18**

Chart 4

XIV → Br_2 → XV → IV-1 → XVI → Zn–$TiCl_4$ → XVII → NaOH → **19**

Chart 5

Chart 6

22: $X_2 = NH_2$

23: $X_2 = NHMe$

24: $X_2 = NMe_2$

Chart 7

Chart 8

26-27, 36-37, 44-45

R_1 = H, F, Cl; R_3 = Me, Et

Chart 9

ing silyl enol ether. 2-[4-(4-Vinylthiazol-2-yl)phenyl]propionic acid (**18**), was prepared by the dehydrochlorination of chloroethyl thiazole XIII, which was prepared by the reaction of 1,4-dichlorobutan-2-one XII with thioamide IV-1 (Chart 4). 1,4-Dichlorobutan-2-one XII, was also prepared by the diazomethane-mediated chloromethylation[12] of 3-chloropropionyl chloride. 2-[4-(4-Allylthiazol-2-yl)phenyl]propionic acid (**19**) was prepared by debromination[14] and a subsequent hydrolysis of diethyl 2-[4-[4-(2,3-dibromopropyl)thiazol-2-yl]phenyl]-2-methylmalonate (XVI), which was prepared by the reaction of 1-chloro-4,5-dibromopentan-2-one, XV, with thioamide IV-1 (Chart 5). Compound XV was prepared by the bromination of 1-chloro-4-penten-3-one, XIV.[15] The compounds with CH_2OH (**20**) and CH_2OMe (**21**) as R_2 substituents were synthesized by alkali hydrolysis or methanolysis followed by hydrolysis of diethyl 2-[4-(4-chloromethylthiazol-2-yl)phenyl]-2-methylmalonate (XVIII), which was prepared by the reaction of 1,3-dichloropropan-2-one with thioamide IV-1 (Chart 6). The compounds with $CONH_2$ (**22**), CONHMe (**23**), or $CON(Me)_2$ (**24**) as R_2 substituents were synthesized by the amidation of diethyl 2-[4-(4-carboxythiazol-2-yl)phenyl]-2-methylmalonate (XIX), which was prepared by the reaction of 3-bromopyruvic acid with thioamide IV-1 (Chart 7). The compound with CF_3 as its R_2 substituent, **25**, was synthesized by hydrolysis and a subsequent dehydration of diethyl 2-methyl-2-[4-(4-trifluoromethyl-4-hydroxy-2-thiazolin-2-yl)phenyl]malonate (XX), which was prepared by the reaction of 1,1,1-trifluoromethyl-3-bromopropan-2-one with thioamide IV-1 (Chart 8).

The derivatives with methyl or ethyl as their R_3 substituent (**26**—**27**, **36**—**37**, and **44**—**45**) were synthesized by the method used for the preparation of **1**—**8** (Chart 9). α-Halo-acetals used in these reactions were synthesized by the method of Rasmussen and Bøwadt.[16]

The compounds synthesized here and their mp and analytical data are shown in Table I.

Results and Discussion

Inhibitory activity of each compound was examined in terms of the 50% inhibitory concentration, I_{50}, for the production of thromboxane B_2 and prostaglandin E_2 from arachidonic acid in the $10000 \times g$ supernatant fraction from guinea pig polymorphonuclear leukocytes by use of a procedure reported elsewhere.[17] The degree of inhibition of the enzyme by the compounds is listed in Table I. The anti-inflammatory activity of the compounds that strongly inhibited cyclooxygenase was evaluated in terms of effects on carrageenin-induced paw edema in rats as described in

TABLE I. Physicochemical and Biological Data of 2-[4-(Thiazol-2-yl)phenyl]propionic Acid Derivatives

No.	R_1	R_2	R_3	mp (°C)[a]	Recrystn. solvent[b]	Formula	Analysis (%) Calcd (Found)			I_{50}[c] (M)
							C	H	N	
1	H	H	H	149—151	A	$C_{12}H_{11}NO_2S$	61.78	4.75	6.00	2.75×10^{-6}
							(61.80	4.58	5.88)	
2	F	H	H	152—154	B	$C_{12}H_{10}FNO_2S$	57.36	4.01	5.57	2.50×10^{-7}
							(57.18	3.93	5.60)	
3	Cl	H	H	81— 82	C	$C_{12}H_{10}ClNO_2S$	53.84	3.77	5.23	2.00×10^{-7}
							(53.75	3.63	5.19)	
4	Br	H	H	137—138	B	$C_{12}H_{10}BrNO_2S$	46.17	3.23	4.49	1.00×10^{-7}
							(46.13	3.13	4.35)	
5	CF_3	H	H	103—105	A	$C_{13}H_{10}F_3NO_2S$	51.83	3.35	4.65	3.00×10^{-7}
							(51.91	3.27	4.57)	
6	Me	H	H	82.5— 84	B	$C_{13}H_{13}NO_2S$	63.14	5.30	5.66	2.00×10^{-6}
							(63.04	5.11	5.54)	
7	OMe	H	H	Liquid		$C_{13}H_{13}NO_3S$	59.30	4.98	5.32	5.20×10^{-6}
							(59.13	5.08	5.04)	
8	SMe	H	H	84— 85	B	$C_{13}H_{13}NO_2S_2$	55.89	4.69	5.01	3.00×10^{-7}
							(55.73	4.58	4.87)	
9	OH	H	H	107.5—108.5	B	$C_{12}H_{11}NO_3S$		250.0537[f]		6.00×10^{-7}
								(250.0503)		
10	NO_2	H	H	111—113	B	$C_{12}H_{10}N_2O_4S$	51.79	3.62	10.07	2.90×10^{-6}
							(51.80	3.54	9.94)	
11	NH_2	H	H	165.5—167	B	$C_{12}H_{12}N_2O_2S$	58.05	4.87	11.28	1.00×10^{-6}
							(58.02	4.77	11.55)	
12	H	Me	H	181—182	B	$C_{13}H_{13}NO_2S$		248.0744		7.20×10^{-7}
								(248.0711)		
13	H	Et	H	125—127	B	$C_{14}H_{15}NO_2S$	64.34	5.79	5.36	3.00×10^{-6}
							(64.29	5.59	5.27)	
14	H	iso-Pr	H	146—148	A	$C_{15}H_{17}NO_2S$	65.43	6.22	5.09	1.63×10^{-5}
							(65.14	6.20	4.95)	
15	H	Bu	H	73—74.5	A	$C_{16}H_{19}NO_2S$	66.41	6.62	4.84	1.05×10^{-4}
							(66.37	6.67	4.87)	
16	H	Acetyl	H	155.5—156.5	A	$C_{14}H_{13}NO_3S$	61.08	4.76	5.09	1.05×10^{-4}
							(60.93	4.65	5.16)	
17	H	Ph	H	157.5—159	B	$C_{18}H_{15}NO_2S$	69.88	4.89	4.53	3.60×10^{-5}
							(70.01	4.80	4.54)	
18	H	Vinyl	H	114—115	B	$C_{14}H_{13}NO_2S$		260.0744		2.10×10^{-6}
								(260.0705)		
19	H	Allyl	H	Amorph.	B	$C_{15}H_{15}NO_2S$	65.91	5.53	5.12	1.30×10^{-5}
							(65.62	5.46	5.02)	
20	H	CH_2OH	H	132—134	B	$C_{13}H_{13}NO_3S$	59.30	4.98	5.32	1.60×10^{-5}
							(59.45	4.92	5.25)	
21	H	CH_2OMe	H	98—100	B	$C_{14}H_{15}NO_3S$	60.63	5.45	5.05	3.00×10^{-5}
							(60.36	5.27	4.94)	
22	H	$CONH_2$	H	245—247	D	$C_{13}H_{12}N_2O_3S$	56.51	4.38	10.14	7.50×10^{-4}
							(56.40	4.38	9.89)	
23	H	CONHMe	H	188—189	E	$C_{14}H_{14}N_2O_3S$	57.92	4.86	9.65	1.02×10^{-3}
							(58.11	4.86	9.63)	
24	H	$CONMe_2$	H	155.5—158	B	$C_{15}H_{16}N_2O_3S$	59.19	5.30	9.20	9.20×10^{-4}
							(58.91	5.20	9.16)	
25	H	CF_3	H	142—144	B	$C_{13}H_{10}F_3NO_2S$	51.83	3.35	4.65	8.20×10^{-6}
							(51.75	3.17	4.54)	
26	H	H	Me	159—160.5	A	$C_{13}H_{13}NO_2S$		248.0744		5.50×10^{-6}
								(248.0713)		
27	H	H	Et	130—132	A	$C_{14}H_{15}NO_2S$	64.34	5.79	5.36	3.20×10^{-6}
							(64.28	5.75	5.26)	
28	H	Me	Me	173.5—175.5	F	$C_{14}H_{15}NO_2S$	64.34	5.79	5.36	1.00×10^{-6}
							(64.34	5.77	5.33)	
29	H	Et	Me	144—145	A	$C_{15}H_{17}NO_2S$	65.43	6.22	5.09	1.02×10^{-5}
							(65.15	6.28	5.11)	
30	H	CH_2CH_2[d]	CH_2[d]	194.5—195.5	E	$C_{15}H_{15}NO_2S$	65.91	5.53	5.12	7.00×10^{-7}
							(65.65	5.40	4.89)	
31	H	CH_2CH_2[e]	CH_2CH_2[e]	187—188	A	$C_{16}H_{17}NO_2S$		288.1057		7.00×10^{-7}
								(288.1094)		
32	F	Me	H	141—143	A	$C_{13}H_{12}FNO_2S$	58.86	4.56	5.28	1.00×10^{-7}
							(58.91	4.48	5.29)	
33	F	Et	H	114—116	C	$C_{14}H_{14}FNO_2S$	60.20	5.05	5.01	1.20×10^{-6}
							(60.08	5.22	5.03)	

TABLE I. (continued)

No.	R_1	R_2	R_3	mp (°C)[a]	Recrystn. solvent[b]	Formula	Analysis (%) Calcd (Found) C	H	N	I_{50}[c] (M)
34	F	iso-Pr	H	116—117.5	A	$C_{15}H_{16}FNO_2S$	61.42 (61.43	5.50 5.50	4.78 4.74)	3.40×10^{-5}
35	F	Bu	H	60— 62	A	$C_{16}H_{18}FNO_2S$	62.52 (62.40	5.90 5.86	4.56 4.45)	5.10×10^{-5}
36	F	H	Me	113—113.5	G	$C_{13}H_{12}FNO_2S$	58.86 (58.58	4.56 4.50	5.28 5.16)	3.00×10^{-7}
37	F	H	Et	118.5—119	A	$C_{14}H_{14}FNO_2S$	60.20 (60.10	5.05 5.13	5.01 5.01)	9.00×10^{-7}
38	F	Me	Me	163.5—164.5	C	$C_{14}H_{14}FNO_2S$	60.20 (60.39	5.05 4.95	5.01 4.96)	2.00×10^{-7}
39	F	Et	Me	136—137.5	A	$C_{15}H_{16}FNO_2S$	61.42 (61.16	5.50 5.45	4.78 4.78)	4.20×10^{-6}
40	Cl	Me	H	141—143	C	$C_{13}H_{12}ClNO_2S$	55.42 (55.39	4.29 4.16	4.97 5.04)	1.00×10^{-7}
41	Cl	Et	H	111—112	C	$C_{14}H_{14}ClNO_2S$	56.85 (57.00	4.77 4.78	4.74 4.79)	7.00×10^{-7}
42	Cl	iso-Pr	H	101—102	A	$C_{15}H_{16}ClNO_2S$	310.0668 (310.0707	312.0638 312.0643)		3.40×10^{-5}
43	Cl	Bu	H	88—89	C	$C_{16}H_{18}ClNO_2S$	59.34 (59.20	5.60 5.59	4.33 4.40)	6.20×10^{-5}
44	Cl	H	Me	106—107	C	$C_{13}H_{12}ClNO_2S$	55.42 (55.35)	4.29 4.34	4.97 4.97)	6.00×10^{-8}
45	Cl	H	Et	120—121	A	$C_{14}H_{14}ClNO_2S$	56.85 (56.71	4.77 4.66	4.74 4.77)	2.00×10^{-7}
46	Cl	Me	Me	158—159	C	$C_{14}H_{14}ClNO_2S$	56.85 (56.80	4.77 4.77	4.74 4.75)	8.00×10^{-7}
47	Cl	Et	Me	102—103	C	$C_{15}H_{16}ClNO_2S$	58.15 (58.29	5.21 5.08	4.52 4.45)	1.20×10^{-6}
	Ibuprofen									1.01×10^{-6}
	Indomethacin									1.00×10^{-7}

a) Compounds that did not show a sharp mp are denoted 'amorph.' *b)* Solvents: A, chloroform–hexane; B, AcOEt–hexane; C, ether–hexane; D, water; E, chloroform–hexane–methanol; F, AcOEt; G, AcOEt–chloroform–hexane. *c)* Concentration for 50% inhibition of cyclooxygenase from guinea pig leukocytes. Each value represents the mean of at least two experiments. *d)* The R_2 and R_3 substituents form a cyclopentane ring. *e)* The R_2 and R_3 substituents form a cyclohexane ring. *f)* High mass data. The upper value was calculated and the lower one was found. The values are for $M+H^+$ (measured by the SI-MS-positive mode).

TABLE II. Anti-inflammatory Activity of 2-[4-(Thiazol-2-yl)phenyl]propionic Acid Derivatives

Compound	Anti-edematous effect ED_{40}[a] (mg/kg)	Cyclooxygenase inhibition I_{50}[b] (M)
1	13	2.8×10^{-6}
3	3	2.0×10^{-7}
8	343	3.0×10^{-7}
32	4	1.0×10^{-7}
36	19	3.0×10^{-7}
38	8	2.0×10^{-7}
40	4	1.0×10^{-7}
44	112	6.0×10^{-8}
Ibuprofen	15	1.0×10^{-6}
Indomethacin	2	1.0×10^{-7}

a) 40% Effective dose. *b)* 50% inhibitory concentration.

the Experimental section. The tested compounds were given orally. Their anti-edematous effect was expressed as the 40% effective dose (ED_{40}, as mg/kg; Table II).

When R_2 and R_3 were not substituted, the effect on cyclooxygenase inhibition of the substituent R_1 in the benzene ring was to increase inhibition by halogens **2**—**4**, trifluoromethyl **5**, methylthio **8**, and hydroxy **9**. There was little change in the inhibition with methyl **6**, methoxy **7**, nitro **10**, and amino **11**. When R_1 and R_3 were not substituted and R_2 was methyl **12**, inhibition was greater than with unsubstituted **1**, but compounds with an R_2 group bulkier than ethyl **13** or vinyl **18** (isopropyl **14**, butyl **15**, acetyl **16**, phenyl **17**, and allyl **19**) caused weaker inhibition. The activity of compounds where R_2 was hydroxymethyl (**20**), methoxymethyl (**21**), amide (**22**—**24**), or trifluoromethyl (**25**) was low. Compound **29**, in which R_2 is ethyl and R_3 is methyl, caused less inhibition than the fused-ring analogs **30** and **31**, which were about four times as active as the unsubstituted **1**. Introduction of fluorine (**32**—**39**) and chlorine (**40**—**47**) into the R_1 position increased inhibition compared with the corresponding compounds lacking an R_1 substituent except when isopropyl was the R_2 substituent, in compounds **34** and **42**, which had about half the activity of the R_1 unsubstituted compound **14**. In general, inhibition increased 2—10 times when fluorine or chlorine was introduced into the R_1 substituent. In particular, compound **44**, in which R_1 is Cl, R_2 is H, and R_3 is Me, caused more inhibition than indomethacin. The effect of the R_1 substituent was not directly linked with any physicochemical substituent property in an obvious pattern, but some steric effect requiring a limited size seemed to be important for the R_2 substituent. That the introduction of halogens into the R_1 position increased inhibition by about the same extent could mean

that the effects of the R_1 and R_2 substituents were additive. Some other factor may affect the activity of compounds in which R_2 is isopropyl.

The anti-inflammatory activity of compounds **3**, **8**, **32**, **36**, **38**, **40**, and **44**, which caused inhibition about 10 times as strong as that of the unsubstituted compound **1**, was measured with compound **1** as the reference (Table II). Compounds **3**, **32**, and **40** had strong anti-inflammatory activity, comparable to that of indomethacin, but the anti-inflammatory activity of **8** (R_1 = SMe and $R_2 = R_3$ = H) and **44** (R_1 = Cl, R_2 = H, and R_3 = Me) was very low despite their strong inhibition of cyclooxygenase. The potent anti-inflammatory activity of **3**, **32**, and **40** matched the strength of their inhibition of cyclooxygenase. The low anti-inflammatory activity of **8** may be because the thiomethyl group is unstable *in vivo*. The reason for the low activity of **44** is not known, but some pharmacokinetic property may be involved, because the R_2-unsubstituted compounds **36** and **44** both had low activity. The results indicated that factors controlling pharmacokinetic properties and the metabolic stability of the molecule[1] could explain relationships between cyclooxygenase inhibition and carrageenin-induced paw edema in rats. The cyclooxygenase inhibition and anti-inflammatory activity of ibuprofen, which are shown in Table II, are lower than those of our selected compounds (Table II). Thiazole moiety should be important in exhibiting not only potent cyclooxygenase inhibition but also potent anti-inflammatory activity. A detailed pharmacological study is in progress.

Experimental

Cyclooxygenase Inhibition Inhibition of cyclooxygenase was assayed as described previously.[17]

Carrageenin Edema in Rat Paw The experiment with rats was done by the method of Winter *et al.*[18] with five or six Sprague–Dawley rats per group, starved for 24 h beforehand. Thirty minutes after the administration of water (4 ml), the test drugs were administered orally. After another 30 min, 0.1 ml of carrageenin was injected subcutaneously into the plantar surface of the left hind paw, and 3h later, the volume of the edema was measured. The anti-edematous effect of the drugs was expressed as a 40% effective dose (ED_{40}).

Analyses Melting points were determined with a Yanaco melting point apparatus and are uncorrected. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Bruker AC-200 NMR spectrometer with tetramethylsilane as the internal standard; chemical shifts are given on the δ (ppm) scale. Infrared (IR) spectra were obtained on a Shimadzu IR-420 spectrometer.

Syntheses: 2-[4-(Thiazol-2-yl)phenyl]propionic Acid (1) 1) Diethyl 2-Methyl-2-(4-nitrophenyl)malonate (II-1): To a suspension of NaH (12.0 g, 60% in mineral oil) in *N*,*N*-dimethylformamide (200 ml, DMF), diethyl methylmalonate (49.8 g) in DMF (20 ml) was added dropwise for 1 h at 0°C. After stirring of the mixture for 30 min at room temperature, 1-chloro-4-nitrobenzene (40.9 g) in DMF (80 ml) was added dropwise for 20 min and the resultant solution was stirred at room temperature for 30 min and then at 90 °C for 2 h. The solution was then cooled to room temperature, poured into ice-water and extracted with hexane–ether (1 : 1). The extract was washed with water and brine, in that order, and dried over $MgSO_4$. After evaporation of the solvent, II-1 was collected by distillation under reduced pressure (43.6 g, 57%), bp 159—161 °C/0.3 mmHg. IR (neat): 3015, 1730, 1605, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.27 (6H, t, J=7.1 Hz), 1.90 (3H, s), 4.26 (4H, q, J=7.1 Hz), 7.58 (2H, d, J=9.1 Hz), 8.21 (2H, d, J=9.1 Hz).

2) Diethyl 2-(4-Cyanophenyl)-2-methylmalonate (III-1): Compound II-1 (10.5 g) in ethyl acetate (100 ml, AcOEt) was hydrogenated over 10% palladium on carbon (1.9 g, Pd–C) under atmospheric pressure at room temperature for 4 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was used in the next step. To a cooled (3 °C) mixture of the amine obtained (9.2 g) in water (70 ml), 12 N HCl (9.8 ml) was added for 5 min. After the mixture was stirred for 10 min, sodium nitrite (2.6 g) in water (15 ml) was added dropwise for 15 min to maintain the temperature of the solution at 2—3 °C. Stirring was continued for another 20 min. Then sodium carbonate (2.6 g) in water (20 ml) was added to adjust the pH of the solution to about 6. The solution obtained was added dropwise for 10 min at 3 °C to a solution of potassium dicyanocuprate prepared by the mixture of cuprous cyanide (6.1 g) and potassium cyanide (8.9 g) in water (70 ml). The mixture was left for 1.5 h at 3 °C and then at 50 °C for 2 h. After being cooled to room temperature, the mixture was poured into water and extracted with AcOEt. The extract was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give III-1 (5.8 g, 60%). IR (neat): 2200, 1725, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.87 (3H, s), 4.25 (4H, q, J=7.1 Hz), 7.51 (2H, d, J=8.7 Hz), 7.65 (2H, d, J=8.7 Hz).

3) Diethyl 2-Methyl-2-(4-thiocarbamoylphenyl)malonate (IV-1): To a solution of compound III-1 (5.7 g) in DMF (70 ml) containing triethylamine (3.5 ml), hydrogen sulfide was bubbled at 90 °C for 30 min and the solution was then stirred at 90 °C for 30 min. After being cooled to room temperature, the reaction mixture was poured into water and extracted with AcOEt. The extract was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane and recrystallized from chloroform–hexane to give IV-1 (6.0 g, 93%). IR (KBr): 3090, 1725, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.2 Hz), 1.87 (3H, s), 4.24 (4H, q, J=7.2 Hz), 7.22 (1H, NH), 7.42 (2H, d, J=8.7 Hz), 7.64 (1H, NH), 7.84 (2H, d, J=8.7 Hz).

4) Diethyl 2-Methyl-2-[4-(thiazol-2-yl)phenyl]malonate (V-1): A solution of IV-1 (1.93 g) and bromoacetaldehyde diethylacetal (1.56 g) in acetic acid (10 ml) containing *p*-toluenesulfonic acid (75 mg) was heated at 100 °C for 1.5 h. After removal of the solvent under reduced pressure, the residue was diluted with AcOEt. The organic layer was washed with saturated sodium bicarbonate solution and brine, and dried over $MgSO_4$, in that order. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give V-1 (1.6 g, 78%). IR (neat) cm^{-1} 1720, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.90 (3H, s), 4.25 (4H, q, J=7.1 Hz), 7.33 (1H, d, J=3.3 Hz), 7.47 (2H, d, J=8.6 Hz), 7.87 (1H, d, J=3.3 Hz), 7.95 (2H, d, J=8.6 Hz).

5) 2-[4-(Thiazol-2-yl)phenyl]propionic Acid (**1**): A solution of V-1 (1.5 g) in ethanol (10 ml) containing KOH (0.99 g) was heated at reflux for 1.5 h. After removal of the solvent, the residue was dissolved in water (100 ml) and the solution obtained was washed with AcOEt. The aqueous layer was cooled to 0 °C, acidified to pH 1 with 12 N HCl and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane and recrystallized from chloroform–hexane to give **1** (0.56 g, 53%). IR (KBr): 3150—2150, 1690, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.54 (3H, d, J=7.2 Hz), 3.79 (1H, q, J=7.2 Hz), 7.32 (1H, d, J=3.3 Hz), 7.40 (2H, d, J=8.3 Hz), 7.7—8.2 (4H, m).

2-[3-Fluoro-4-(thiazol-2-yl)phenyl]propionic Acid (2) The procedure used for the preparation of **1** was repeated with 2,4-difluoronitrobenzene as a starting material and dimethylsulfoxide was used as a solvent for the synthesis of diethyl 2-(3-fluoro-4-nitrophenyl)-2-methylmalonate. Yields of I-2 to II-2, II-2 to III-2, III-2 to IV-2, IV-2 to V-2, and V-2 to **2** were 80, 57, 83, 45, and 41%, respectively. The spectra of compound **2** were as follows. IR (KBr): 3100—2300, 1700, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.45 (3H, d, J=7.1 Hz), 3.77 (1H, q, J=7.1 Hz), 7.2—7.3 (2H, m), 7.7—7.8 (1H, m), 7.9—8.0 (1H, m), 8.1—8.3 (1H, m).

2-[3-Chloro-4-(thiazol-2-yl)phenyl]propionic Acid (3) The procedure used for the preparation of **1** was repeated with 2,4-dichloronitrobenzene as a starting material. Yields of I-3 to II-3, II-3 to III-3, III-3 to IV-3, IV-3 to V-3, and V-3 to **3** were 53, 63, 98, 75, and 84% respectively. The spectra of compound **3** were as follows. IR (KBr): 3250—2200, 1690, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.54 (3H, d, J=7.2 Hz), 3.76 (1H, q, J=7.2 Hz), 7.33 (1H, dd, J=8.2, 1.8 Hz), 7.47 (1H, d, J=1.8 Hz), 7.50 (1H, d, J=3.4 Hz), 7.97 (1H, d, J=3.4 Hz), 8.09 (1H, d, J=8.2 Hz), 7.7—8.6 (1H, OH).

2-[3-Bromo-4-(thiazol-2-yl)phenyl]propionic Acid (4) The procedure used for the preparation of **1** was repeated with 2-bromo-4-chloronitrobenzene. 2-Bromo-4-chloronitrobenzene was prepared by the reported method[9] by use of cuprous bromide (93%). Yields of I-4 to II-4, II-4 to III-4, III-4 to IV-4, IV-4 to V-4, and V-4 to **4** were 51, 9, 86, 80, and 71%, respectively. The spectra of compound **4** were as follows. IR (KBr): 3100—2320, 1700, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.42 (3H, d,

J=7.2 Hz), 3.80 (1H, q, J=7.2 Hz), 7.45 (1H, dd, J=8.2, 1.7 Hz), 7.73 (1H, d, J=1.7 Hz), 7.92 (1H, d, J=3.2 Hz), 7.9—8.1 (2H, m), 11.7—13.0 (1H, br, OH).

2-[4-(Thiazol-2-yl)-3-trifluoromethylphenyl]propionic Acid (5) The procedure used for the preparation of **1** was repeated with 4-chloro-2-trifluoromethylnitrobenzene as a starting material. Yields of I-5 to II-5, II-5 to III-5, III-5 to IV-5, IV-5 to V-5, and V-5 to **5** were 97, 41, 54, 48, and 53% respectively. The spectra of compound **5** were as follows. IR (KBr): 3200—2300, 1720, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.44 (3H, d, J=7.1 Hz), 3.95 (1H, q, J=7.1 Hz), 7.71 (2H, m), 7.82 (1H, m), 7.93 (1H, d, J=3.2 Hz), 7.98 (1H, d, J=3.2 Hz), 12.6 (1H, OH).

2-[3-Methyl-4-(thiazol-2-yl)phenyl]propionic Acid (6) The procedure used for preparation of **1** was repeated with 4-fluoro-2-methylnitrobenzene as a starting material. Yields of I-6 to II-6, II-6 to III-6, III-6 to IV-6, IV-6 to V-6, and V-6 to **6** were 82, 71, 69, 90, and 37%, respectively. The spectra of compound **6** were as follows. IR (KBr): 3000—2300, 1700, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.52 (3H, d, J=7.2 Hz), 2.53 (3H, s), 3.74 (1H, q, J=7.2 Hz), 7.2—7.3 (2H, m), 7.39 (1H, d, J=3.3 Hz), 7.64 (1H, d, J=7.8 Hz), 7.93 (1H, d, J=3.3 Hz).

2-[3-Methoxy-4-(thiazol-2-yl)phenyl]propionic Acid (7) The procedure used for preparation of **1** was repeated with 4-fluoro-2-methoxynitrobenzene as a starting material. 4-Fluoro-2-methoxynitrobenzene was synthesized as follows. A mixture of 4-fluoro-2-hydroxynitrobenzene (9.2 g), tetrabutylammonium hydrogen sulfate (0.99 g), 1 N NaOH (61 ml), dichloromethane (61 ml), and dimethylsulfate (6.1 ml) was stirred at room temperature for 67 h. After the reaction, the mixture was diluted with dichloromethane. The organic layer was washed with water and brine, and dried over $MgSO_4$, in that order. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give 4-fluoro-2-methoxynitrobenzene I-7 (9.4 g, 94%). IR (KBr): 1620, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 3.98 (3H, s), 6.74 (1H, m), 6.82 (1H, m), 7.96 (1H, m).

Yields of I-7 to II-7, II-7 to III-7, III-7 to IV-7, IV-7 to V-7, and V-7 to **7** were 93, 90, 63, 58, and 98% respectively. The spectra of compound **7** were as follows. IR ($CHCl_3$) cm^{-1}: 3200—2800, 1710, 1600 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.55 (3H, d, J=7.2 Hz), 3.78 (1H, q, J=7.2 Hz), 3.99 (3H, s), 6.99 (1H, d, J=1.7 Hz), 7.05 (1H, dd, J=8.1, 1.7 Hz), 7.37 (1H, d, J=3.3 Hz), 7.92 (1H, d, J=3.3 Hz), 8.24 (1H, d, J=8.1 Hz), 9.5 (1H, OH).

2-[3-Methylthio-4-(thiazol-2-yl)phenyl]propionic Acid (8) 1) Diethyl 2-Methyl-2-(3-methylthio-4-nitrophenyl)malonate (II-8): To a solution of dimethyldisulfide (3.4 ml) in ethanol (85 ml) and water (10 ml) was added tri-*n*-butylphosphine (9.8 ml) for 10 min at 0 °C. After stirring of the mixture of 30 min at 0 °C and 70 min at room temperature, diethyl 2-(3-chloro-4-nitrophenyl)-2-methylmalonate (II-3, 8.5 g) in ethanol (50 ml) and 1 N NaOH (38 ml) were added to the solution one after another, each taking 15 min at 0 °C. The solution was stirred for 40 min at 0 °C and then for 4.1 h at room temperature. The solution was extracted with AcOEt and the organic layer was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give compound II-8 (7.5 g, 86%). IR (KBr): 1720, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.28 (6H, t, J=7.2 Hz), 1.90 (3H, s), 2.49 (3H, s), 4.26 (4H, q, J=7.2 Hz), 7.26 (1H, dd, J=8.8, 2.0 Hz), 7.45 (1H, d, J=2.0 Hz), 8.23 (1H, d, J=8.8 Hz). The procedure used for preparation of **1** was repeated with 2-methyl-2-(3-methylthio-4-nitrophenyl)malonate II-8. Yields of II-8 to III-8, III-8 to IV-8, IV-8 to V-8, and V-8 to **8** were 60, 96, 80, and 60% respectively. The spectra of compound **8** were as follows. IR(KBr): 3600—2300, 1710, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.47 (3H, d, J=7.1 Hz), 2.48 (3H, s), 3.79 (1H, q, J=7.1 Hz), 7.21 (1H, dd, J=8.1, 1.6 Hz), 7.36 (1H, d, J=1.6 Hz), 7.80 (1H, d, J=8.1 Hz), 7.83 (1H, d, J=3.3 Hz), 7.96 (1H, d, J=3.3 Hz), 11—13 (1H, OH).

2-[3-Hydroxy-4-(thiazol-2-yl)phenyl]propionic Acid (9) To a mixture of aluminum chloride[10] (18.7 g), sodium iodide (21.1 g) in dry acetonitrile (270 ml), and dry dichloromethane (135 ml), **7** (1.85 g) in acetonitrile (10 ml) and dichloromethane (5 ml) was added for 10 min at 0 °C. The reaction mixture was stirred at reflux for 11 h, poured into ice-water, and extracted with AcOEt. The organic layer was washed with 1 N sodium thiosulfate solution, water, and brine, in that order, and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with chloroform–methanol and recrystallized from AcOEt–hexane to give compound **9** (1.05 g, 60%). IR (KBr): 3300—2500, 2700, 1685, 1620, 1585 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.38 (3H, d, J=7.1 Hz), 3.67 (1H, q, J=7.1 Hz), 6.89 (1H, dd, J=8.1, 1.5 Hz), 6.97 (1H, d, J=1.5 Hz), 7.73 (1H, d, J=3.3 Hz), 7.92 (1H, d, J=3.3 Hz), 7.99 (1H, d, J=8.1 Hz), 11—12 (1H, OH), 12—13 (1H, OH).

2-[3-Nitro-4-(thiazol-2-yl)phenyl]propionic Acid (10) 1) Methyl 4-Carbamoyl-3-nitrobenzoate (VII): A solution of nitroterephthalic acid (20.0 g) in dry methanol (100 ml) and concentrated sulfuric acid (10 ml) was stirred at reflux for 1 h. After evaporation of the solvent, the residue was poured into $NaHCO_3$ and the aqueous layer was washed with chloroform. After the solution was acidified to pH 2 with 2 N HCl, the solution was extracted with AcOEt. The organic layer was washed with water and brine, in that order, and over $MgSO_4$. After evaporation of the solvent, the residue was recrystallized from AcOEt–hexane to give the ester (14.7 g), mp 132—133 °C. A solution of the ester (7.2 g) in thionyl chloride (62 ml) containing 2—3 drops of DMF was stirred at reflux for 3 h. After removal of excess thionyl chloride by distillation, the residue (7.83 g) was used in the next step. To a solution of 28% ammonium hydroxide (30 ml) and 1 N NaOH (3 ml) was added the acid chloride (6.88 g) in dichloromethane (30 ml), and the mixture was stirred at room temperature for 1.5 h. The precipitated solid was collected by filtration and dried to give VII (5.9 g, 64%). IR (KBr): 3360, 3100, 1720, 1660, 1540 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 3.93 (3H, s), 7.78 (1H, d, J=7.9 Hz), 7.86 (1H, NH), 8.28 (1H, NH), 8.29 (1H, dd, J=7.9, 1.6 Hz), 8.44 (1H, d, J=1.6 Hz).

2) Methyl 3-Nitro-4-(thiazol-2-yl)benzoate (VIII): A solution of VII (4.95 g) and phosphorous pentasulfide (2.0 g) in dioxane (200 ml) was stirred at reflux for 1 h. After filtration of the reaction mixture, the filtrate was concentrated under reduced pressure. The residue obtained was chromatographed on silica gel with AcOEt–hexane to give thioamide (4.35 g). The thioamide was converted by the procedure used for the synthesis of V-1 to give VIII (2.6 g, 46%). IR (KBr): 3100, 1715, 1540 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 4.00 (3H, s), 7.54 (1H, d, J=3.2 Hz), 7.86 (1H, d, J=8.0 Hz), 7.96 (1H, d, J=3.2 Hz), 8.29 (1H, dd, J=8.0, 1.6 Hz), 8.43 (1H, d, J=1.6 Hz).

3) Benzyl 2-[3-Nitro-4-(thiazol-2-yl)phenyl]propionate (IX): The methyl ester (2.61 g) was hydrolyzed by a procedure similar to the synthesis of **1**, giving the benzoic acid (2.2 g). 3-Nitro-4-(thiazol-2-yl)benzoyl chloride was prepared quatitatively by a procedure similar to the synthesis of methyl 4-chloroformyl-3-nitrobenzoate. The acid chloride was converted to IX by a method reported elsewhere.[11] The residue obtained was chromatographed on silica gel with AcOEt–hexane to give IX (1.05 g, 43%). IR (neat): 1715, 1530 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.58 (3H, d, J=7.2 Hz), 3.89 (1H, q, J=7.2 Hz), 5.14 (2H, m), 7.2—7.4 (5H, m), 7.47 (1H, d, J=3.3 Hz), 7.56 (1H, dd, J=8.0, 1.8 Hz), 7.68 (1H, d, J=8.0 Hz), 7.75 (1H, d, J=1.8 Hz), 7.90 (1H, d, J=3.3 Hz).

4) 2-[3-Nitro-4-(thiazol-2-yl)phenyl]propionic Acid (**10**): The procedure used for the preparation of **1** was repeated with IX (1.15 g) to obtain **10** (610 mg, 71%). IR (KBr): 3150—2200, 1725, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.46 (3H, d, J=7.2 Hz), 3.94 (1H, q, J=7.2 Hz), 7.72 (1H, dd, J=8.1, 1.6 Hz), 7.79 (1H, d, J=8.1 Hz), 7.90 (1H, d, J=1.6 Hz), 7.93 (1H, d, J=3.2 Hz), 7.95 (1H, d, J=3.2 Hz), 12.3—13.1 (1H, OH).

2-[3-Amino-4-(thiazol-2-yl)phenyl]propionic Acid (11) 1) Benzyl 2-[3-Amino-4-(thiazol-2-yl)phenyl]propionate (X): The procedure used for the preparation of III-1 was repeated with IX (1.1 g), and platinum oxide (68 mg) in acetic acid (20 ml) to give X (870 mg, 87%). IR (neat): 3430 3300, 1720, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.50 (3H, d, J=7.2 Hz), 3.69 (1H, q, J=7.2 Hz), 5.1—5.2 (2H, m), 6.12 (2H, NH_2), 6.6—6.7 (2H, m), 7.20 (1H, d, J=3.4 Hz), 7.25—7.35 (5H, m), 7.58 (1H, d, J=8.6 Hz), 7.78 (1H, d, J=3.4 Hz).

2) 2-[3-Amino-4-(thiazol-2-yl)phenyl]propionic Acid (**11**): The procedure used for the preparation of **1** was repeated with X (870 mg). After the reaction, the solution was concentrated under reduced pressure and the aqueous layer obtained was acidified with 4 N HCl to pH 4 and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was recrystallized from AcOEt to give compound **11** (480 mg, 76%). IR (KBr): 3300, 1690, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.34 (3H, d, J=7.1 Hz), 3.54 (1H, q, J=7.1 Hz), 6.53 (1H, dd, J=8.2, 1.6 Hz), 6.75 (1H, d, J=1.6 Hz), 7.00 (2H, NH_2), 7.51 (1H, d, J=8.2 Hz), 7.61 (1H, d, J=3.4 Hz), 7.85 (1H, d, J=3.4 Hz).

2-[4-(4-Methylthiazol-2-yl)phenyl]propionic Acid (12) 1) Diethyl 2-Methyl-2-[4-(4-methylthiazol-2-yl)phenyl]malonate (XI-12): A solution of IV-1 (2.0 g) and chloroacetone (1.32 ml, 90% purity) in dry benzene (40 ml) was stirred at reflux for 3.25 h. The solution was diluted with ether, and the organic layer was washed with water and brine, and dried over $MgSO_4$, in that order. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give XI-12 quantitatively. IR (neat): 1730, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.89 (3H, s), 2.51 (3H, s), 4.24 (4H, q, J=7.1 Hz), 6.67 (1H, s), 7.44 (2H, d, J=8.5 Hz), 7.90 (2H, d, J=8.5 Hz).

TABLE III. Yields, IR, and NMR Data for 2-[4-(Thiazol-2-yl)phenyl]propionic Acid Derivatives

No.	Yield (%)[a]	IR (KBr) cm^{-1}	NMR (δ in DMSO-d_6 from TMS)
13	68	3600—2800 1725	1.27 (3H, t, J=7.5 Hz), 1.41 (3H, d, J=7.1 Hz), 2.79 (1H, dq, J=0.7, 7.5 Hz), 3.76 (1H, q, J=7.1 Hz), 7.29 (1H, t, J=0.7 Hz), 7.4—7.5 (2H, m), 7.8—7.9 (2H, m), 12.4 (s, OH)
14[b]	70	3000—2300 1720	1.30 (6H, d, J=6.8 Hz), 1.40 (3H, d, J=7.1 Hz), 3.08 (1H, m), 3.74 (1H, q, J=7.1 Hz), 7.24 (1H, s), 7.40 (2H, d, J=8.3 Hz), 7.85 (2H, d, J=8.3 Hz), 12.4 (s, OH)
15[b]	14	3000—2300 1720	0.92 (3H, t, J=7.2 Hz), 1.2—1.8 (4H, m), 1.40 (3H, d, J=7.1 Hz), 2.75 (2H, t, J=7.2 Hz), 3.74 (1H, q, J=7.1 Hz), 7.28 (1H, s), 7.40 (2H, d, J=7.9 Hz), 7.85 (2H, d, J=7.9 Hz), 12.4 (s, OH)
16	32	3200—2400 1720	1.41 (3H, d, J=7.1 Hz), 2.64 (3H, s), 3.79 (1H, q, J=7.1 Hz), 7.46 (2H, d, J=8.3 Hz), 7.96 (2H, d, J=8.3 Hz), 8.56 (1H, s), 12.4 (s, OH)
17	40	3600—2700 1690	1.41 (3H, d, J=7.1 Hz), 3.76 (1H, q, J=7.1 Hz), 7.3—7.5 (5H, m), 7.9—8.1 (4H, m), 8.15 (1H, s)
28	53	3600—2800 1700	1.39 (3H, d, J=7.1 Hz), 2.31 (3H, s), 2.36 (3H, s), 3.74 (1H, q, J=7.1 Hz), 7.3—7.4 (2H, m), 7.7—7.8 (2H, m), 12.4 (s, OH)
29[c]	22	3000—2300 1720	1.20 (3H, t, J=7.5 Hz), 1.37 (3H, d, J=7.1 Hz), 2.36 (3H, s), 2.64 (2H, q, J=7.5 Hz), 3.70 (1H, q, J=7.1 Hz), 7.35 (2H, d, J=8.1 Hz), 7.77 (2H, d, J=8.1 Hz), 12.4 (s, OH)
30	70	3000—2300 1700	1.39 (3H, d, J=7.1 Hz), 2.47 (2H, m), 2.81 (2H, t, J=7.1 Hz), 2.92 (2H, t, J=7.1 Hz), 3.73 (1H, q, J=7.1 Hz), 7.37 (2H, d, J=8.3 Hz), 7.85 (2H, d, J=8.3 Hz), 12.4 (s, OH)
31[c]	62	3600—3000 1715	1.39 (3H, d, J=7.1 Hz), 1.83 (4H, m), 2.76 (4H, m), 3.72 (1H, q, J=7.1 Hz), 7.37 (2H, d, J=8.1 Hz), 7.80 (2H, d, J=8.1 Hz), 12.4 (s, OH)
32	87	3000—2300 1715	1.39 (3H, d, J=7.2 Hz), 2.43 (3H, s), 3.78 (1H, q, J=7.2 Hz), 7.2—7.4 (2H, m), 7.41 (1H, s), 8.13 (1H, dd, J=8.1, 8.1 Hz), 12.5 (s, OH)
33	68	3000—2300 1710	1.26 (3H, t, J=7.5 Hz), 1.39 (3H, d, J=7.1 Hz), 2.79 (2H, q, J=7.5 Hz), 3.79 (1H, q, J=7.1 Hz), 7.2—7.4 (2H, m), 7.43 (1H, s), 8.14 (1H, dd, J=8.1, 8.1 Hz), 12.6 (s, OH)
34[b]	64	3000—2300 1720	1.31 (6H, d, J=6.9 Hz), 1.42 (3H, d, J=7.1 Hz), 3.12 (1H, m), 3.80 (1H, q, J=7.1 Hz), 7.2—7.4 (2H, m), 7.40 (1H, s), 8.18 (1H, dd, J=8.0, 8.0 Hz), 12.5 (s, OH)
35[b]	50	3000—2300 1720	0.93 (3H, t, J=7.0 Hz), 1.2—1.7 (4H, m), 1.41 (3H, d, J=7.1 Hz), 2.79 (2H, t, J=7.0 Hz), 3.80 (1H, q, J=7.1 Hz), 7.25—7.35 (2H, m), 7.41 (1H, s), 8.16 (1H, dd, J=8.0, 8.0 Hz), 12.6 (s, OH)
38	70	3000—2300 1705	1.38 (3H, d, J=7.1 Hz), 2.31 (3H, s), 2.36 (3H, s), 3.77 (1H, q, J=7.1 Hz), 7.2—7.4 (2H, m), 8.08 (1H, dd, J=8.1, 8.1 Hz), 12.6 (s, OH)
39[c]	45	3000—2300 1710	1.23 (3H, t, J=7.5 Hz), 1.41 (3H, d, J=7.1 Hz), 2.40 (3H, s), 2.70 (2H, q, J=7.5 Hz), 3.78 (1H, q, J=7.1 Hz), 7.2—7.4 (2H, m), 8.12 (1H, dd, J=8.0, 8.0 Hz), 12.5 (s, OH)
40	59	3000—2300 1710	1.39 (3H, d, J=7.1 Hz), 2.44 (3H, s), 3.79 (1H, q, J=7.1 Hz), 7.39 (1H, dd, J=8.2, 1.5 Hz), 7.47 (1H, s), 7.53 (1H, d, J=1.5 Hz), 8.12 (1H, d, J=8.2 Hz), 12.6 (s, OH)
41	57	3100—2250 1710	1.35 (3H, t, J=7.5 Hz), 1.39 (3H, d, J=7.1 Hz), 2.80 (2H, q, J=7.5 Hz), 3.79 (1H, q, J=7.1 Hz), 7.39 (1H, dd, J=8.2, 1.5 Hz), 7.48 (1H, s), 7.53 (1H, d, J=1.5 Hz), 8.12 (1H, d, J=8.2 Hz), 12.6 (s, OH)
42[b]	52	3050—2300 1720	1.28 (6H, d, J=6.9 Hz), 1.39 (3H, d, J=7.1 Hz), 3.09 (1H, m), 3.79 (1H, q, J=7.1 Hz), 7.40 (1H, m), 7.44 (1H, s), 7.52 (1H, m), 8.12 (1H, d, J=8.2 Hz), 12.6 (s, OH)
43[b]	50	3050—2300 1710	0.89 (3H, t, J=7.3 Hz), 1.33 (2H, m), 1.39 (3H, d, J=7.1 Hz), 1.66 (2H, m), 2.76 (2H, t, J=7.3 Hz), 3.78 (1H, q, J=7.1 Hz), 7.3—7.5 (2H, m), 7.52 (1H, d, J=1.4 Hz), 8.12 (1H, d, J=8.2 Hz), 12.6 (s, OH)
46	43	3000—2300 1710	1.38 (3H, d, J=7.1 Hz), 2.32 (3H, s), 2.38 (3H, s), 3.77 (1H, q, J=7.1 Hz), 7.36 (1H, m), 7.49 (1H, m), 8.09 (1H, d, J=8.2 Hz), 12.6 (s, OH)
47[c]	54	3050—2300 1710	1.20 (3H, t, J=7.5 Hz), 1.39 (3H, d, J=7.1 Hz), 2.39 (3H, s), 2.68 (2H, q, J=7.5 Hz), 3.77 (1H, q, J=7.1 Hz), 7.37 (1H, dd, J=8.2, 1.5 Hz), 7.50 (1H, d, J=1.5 Hz), 8.10 (1H, d, J=8.2 Hz), 12.6 (s, OH)

a) Yields from the corresponding thioamides (IV-1, IV-2, or IV-3). *b)* α-Haloketones used in these reactions were prepared from isobutyryl chloride (for the syntheses of **14**, **34**, and **42**) or valeryl chloride (for the syntheses of **15**, **35**, and **43**) by use of diazomethane-mediated chloromethylation.[12] *c)* α-Haloketones used in these reactions were prepared by the bromination[13] of the silyl enol ether of cyclohexanone (for the synthesis of **31**) or 3-pentanone (for the syntheses of **29**, **39**, and **47**).

2) 2-[4-(4-Methylthiazol-2-yl)phenyl]propionic Acid (**12**): The procedure used for the preparation of **1** was repeated with XI-12 (2.1 g) and 1 N NaOH (25 ml) in ethanol (10 ml) to obtain **12** (1.1 g, 74%). IR (KBr): 3000—2800, 1720, 1600, 1530 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.37 (3H, d, J=7.1 Hz), 2.40 (3H, d, J=0.7 Hz), 3.73 (1H, q, J=7.1 Hz), 7.29 (1H, m), 7.38 (2H, d, J=8.3 Hz), 7.85 (2H, d, J=8.3 Hz), 12.4 (1H, OH). The compounds **13**—**17**, **28**—**35**, **38**—**43**, and **46**—**47** were prepared as in the synthesis of **12**. The yields and physical properties are summarized in Table III.

2-[4-(4-Vinylthiazol-2-yl)phenyl]propionic Acid (18) 1) Diethyl 2-[4-[4-(2-Chloroethyl)thiazol-2-yl]phenyl]-2-methylmalonate (XIII): The procedure used for the preparation of XI-12 was repeated with IV-1 (1.4 g) and 1,4-dichloro-2-butanone (850 mg: prepared from propionyl chloride by a reported method[12]) in dry benzene (25 ml), giving XIII (970 mg). IR(KBr): 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.2 Hz), 1.88 (3H, s), 3.26 (2H, t, J=7.0 Hz), 3.90 (2H, t, J=7.0 Hz), 4.24 (4H, q, J=7.2 Hz), 7.00 (1H, s), 7.45 (2H, d, J=8.6 Hz), 7.90 (2H, d, J=8.6 Hz).

2) 2-[4-(4-Vinylthiazol-2-yl)phenyl]propionic Acid (**18**): The procedure used for the preparation of **12** was repeated with XIII (970 mg) and 1 N NaOH (48.8 ml) in ethanol to obtain **18** (370 mg, 58%). IR (KBr): 3200—2500, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.53 (3H, d, J=7.1 Hz), 3.78 (1H, q, J=7.1 Hz), 5.40 (1H, dd, J=10.8, 1.6 Hz), 6.14 (1H, dd, J=17.3, 1.6 Hz), 6.76 (1H, dd, J=17.3, 10.8 Hz), 7.10 (1H, s), 7.38 (2H, d, J=8.4 Hz), 7.92 (2H, d, J=8.4 Hz).

2-[4-(4-Allylthiazol-2-yl)phenyl]propionic Acid (19) 1) 4,5-Dibromo-1-chloro-2-pentanone (XV): Bromine was added dropwise to a solution of crude 1-chloro-4-penten-2-one (XIV) [prepared from allyltrimethylsilane (1.4 g) and chloroacetonitrile (0.94 ml) by a method published elsewhere[15]] in dichloromethane (100 ml) at −78 °C until the solution turned orange. After the addition ended, the solution was stirred at −60 °C for 30 min. Water added to the solution and the resultant mixture was extracted with ether. The organic layer was washed with $NaHCO_3$, water, and brine, and dried over $MgSO_4$, in that order. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give XV (980 mg, 28% based on chloroacetonitrile). IR (neat): 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 3.26 (1H, dd, J=17.9, 8.8 Hz), 3.53 (1H, dd, J=17.9, 3.9 Hz), 3.71 (1H, dd, J=10.3, 9.7 Hz), 3.93 (1H, dd, J=10.3, 4.3 Hz), 4.14 (2H, s), 4.5—4.6 (1H, m).

2) Diethyl 2-[4-[4-(2,3-Dibromopropyl)thiazol-2-yl]phenyl]-2-methylmalonate (XVI): The procedure used for preparation of XI-12 was repeated with VI-1 (1.4 g) and XV (1.3 g) in dry benzene (30 ml), giving XVI (1.6 g) quantitatively. IR (neat): 1730, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.89 (3H, s), 3.33 (1H, dd, J=14.9, 7.6 Hz), 3.64 (1H, dd, J=14.9, 5.0 Hz), 3.86 (2H, m), 4.24 (4H, q, J=7.1 Hz), 4.68 (1H, m), 7.10 (1H, s), 7.45 (2H, d, J=8.5 Hz), 7.91 (2H, d, J=8.5 Hz).

TABLE IV. Yields, IR, and NMR Data for 2-[4-(5-Alkylthiazol-2-yl)phenyl]propionic Acids

No.	Yield (%)[a]	IR (KBr) cm^{-1}	NMR (δ in DMSO-d_6 from TMS)
26[b]	35	3000—2300 1700	1.39 (3H, d, J=7.1 Hz), 2.49 (3H, s), 3.72 (1H, q, J=7.1 Hz), 7.38 (2H, d, J=8.0 Hz), 7.56 (1H, s), 7.81 (2H, d, J=8.0 Hz)
27[b]	52	2900—2300 1720	1.29 (3H, t, J=7.5 Hz), 1.40 (3H, d, J=7.1 Hz), 2.87 (2H, q, J=7.5 Hz), 3.72 (1H, q, J=7.1 Hz), 7.38 (2H, d, J=8.2 Hz), 7.58 (1H, s), 7.82 (2H, d, J=8.2 Hz), 12.4 (s, OH)
36[b]	45	3000—2300 1700	1.41 (3H, d, J=7.1 Hz), 2.51 (3H, s), 3.80 (1H, q, J=7.1 Hz), 7.25—7.35 (2H, m), 7.67 (1H, s), 8.12 (1H, dd, J=8.0, 8.0 Hz), 12.4 (1H, OH)
37[b]	31	3200—2300 1720	1.31 (3H, t, J=7.7 Hz), 1.42 (3H, d, J=7.1 Hz), 2.91 (2H, q, J=7.7 Hz), 3.79 (1H, q, J=7.1 Hz), 7.25—7.35 (2H, m), 7.69 (1H, s), 8.13 (1H, dd, J=8.0, 8.0 Hz), 12.5 (1H, OH)
44[b]	54	3200—2300 1690	1.40 (3H, d, J=7.1 Hz), 2.50 (3H, s), 3.79 (1H, q, J=7.1 Hz), 7.38 (1H, m), 7.52 (1H, m), 7.68 (1H, s), 8.09 (1H, d, J=8.2 Hz), 12.6 (1H, OH)
45[b]	57	3050—2200 1715	1.27 (3H, t, J=7.5 Hz), 1.39 (3H, d, J=7.1 Hz), 2.90 (2H, q, J=7.5 Hz), 3.78 (1H, q, J=7.1 Hz), 7.38 (1H, m), 7.52 (1H, m), 7.72 (1H, s), 8.08 (1H, d, J=8.3 Hz), 12.6 (1H, OH)

a) Yields from the corresponding thioamides (IV-1, IV-2, or IV-3). *b*) Dimethyl acetal of 2-bromopropanal (for the syntheses of **26**, **36**, and **44**) and dimethyl acetal of 2-bromobutanal (for the syntheses of **27**, **37**, and **45**) were prepared by a reported method.[16]

3) Diethyl 2-[4-(4-Allylthiazol-2-yl)phenyl]-2-methylmalonate (XVII): The reported method[14] was used with XVI (1.0 g), zinc dust (850 mg), and titanium (IV) chloride (0.5 ml) in tetrahydrofuran (10 ml) to give XVII (750 mg of crude product) quantitatively. IR (neat): 1730, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.89 (3H, s), 3.61 (2H, m), 4.23 (4H, q, J=7.1 Hz), 5.1—5.3 (2H, m), 6.0—6.2 (1H, m), 6.92 (1H, s), 7.45 (2H, d, J=8.5 Hz), 7.91 (2H, d, J=8.5 Hz).

4) 2-[4-(4-Allylthiazol-2-yl)phenyl]propionic Acid (**19**): The procedure used for the preparation of **12** was repeated with XVII (1.5 g) and 1 N NaOH (12.3 ml) in ethanol (5 ml) to give **19** (850 mg, 76%). IR (KBr): 3000—2400, 1720, 1510 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.40 (3H, d, J=7.1 Hz), 3.54 (2H, m), 3.74 (1H, q, J=7.1 Hz), 5.1—5.3 (2H, m), 5.9—6.1 (1H, m), 7.27 (1H, s), 7.39 (2H, d, J=8.3 Hz), 7.86 (2H, d, J=8.3 Hz).

2-[4-(4-Hydroxymethylthiazol-2-yl)phenyl]propionic Acid (20) 1) Diethyl 2-[4-(4-Chloromethylthiazol-2-yl)phenyl]-2-methylmalonate (XVIII): The procedure used for the preparation of XI-12 was repeated with IV-1 (3.3 g) and 1,3-dichloroacetone (1.7 g) in dry benzene (50 ml) to give XVIII (3.7 g, 91%). IR (neat): 1720, 1505 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.89 (3H, s), 4.24 (4H, q, J=7.1 Hz), 4.74 (2H, s), 7.30 (1H, s), 7.46 (2H, d, J=8.4 Hz), 7.86 (2H, d, J=8.4 Hz).

2) 2-[4-(4-Hydroxymethylthiazol-2-yl)phenyl]propionic Acid (**20**): The procedure used for the preparation of **12** was repeated with XVIII (5.2 g) and 1 N NaOH (50 ml) in ethanol (10 ml) to give **20** (800 mg, 22%). IR (KBr): 3400, 3300—2500, 1700 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.40 (3H, d, J=7.1 Hz), 3.73 (1H, q, J=7.1 Hz), 4.63 (2H, s), 5.30 (1H, OH), 7.43 (1H, s), 7.41 (2H, d, J=8.2 Hz), 7.87 (2H, d, J=8.2 Hz), 12.5 (1H, OH).

2-[4-(4-Methoxymethylthiazol-2-yl)phenyl]propionic Acid (21) A solution of XVIII (3.2 g) and sodium methoxide (1.4 g) in methanol (50 ml) was stirred at reflux for 2 h. After the reaction, the solution was poured into ice-water and acidified to pH 2 with 1 N HCl. The mixture was extracted with chloroform and the extract was washed with water and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane to give the 4-methoxymethylthiazole derivative (1.9 g, 66%). The compound obtained was treated by a method similar to that for preparation of **12**, giving **21** (1.3 g, 67%). IR (KBr): 3200—2600, 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.54 (3H, d, J=7.1 Hz), 3.49 (3H, s), 3.77 (1H, q, J=7.1 Hz), 4.64 (2H, s), 7.21 (1H, s), 7.37 (2H, d, J=8.2 Hz), 7.87 (2H, d, J=8.2 Hz).

2-[4-(4-Carbamoylthiazol-2-yl)phenyl]propionic Acid (22) 1) Diethyl 2-[4-(4-Carboxythiazol-2-yl)phenyl]-2-methylmalonate (XIX): The procedure used for the preparation of XI-12 was repeated with IV-1 (3.0 g) and 3-bromopyruvic acid (2.1 g) in dry benzene (40 ml) to give XIX (2.6 g, 71%). IR (KBr): 3100—2500, 1720, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.27 (6H, t, J=7.0 Hz), 1.90 (3H, s), 4.24 (4H, q, J=7.0 Hz), 7.50 (2H, d, J=8.6 Hz), 7.97 (2H, d, J=8.6 Hz), 8.29 (1H, s).

2) 2-[4-(4-Carbamoylthiazol-2-yl)phenyl]propionic Acid (**22**): A solution of XIX (1.1 g) and thionyl chloride (3.8 g) in dichloromethane (20 ml) was stirred at reflux for 100 min. After removal of the solvent and thionyl chloride, the residue obtained was added dropwise to 28% ammonium hydroxide (50 ml) at 0 °C. The solution was stirred for 10 min and extracted with AcOEt. The extract was washed with water and brine, and dried over $MgSO_4$, in that order. After evaporation of the solvent, the residue was precipitated from chloroform–hexane. The white solid obtained was converted by a method similar to that used for the preparation of **12** to give **22** (20%). IR (KBr): 3600—3000, 1685, 1640 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.41 (3H, d, J=7.0 Hz), 3.76 (1H, q, J=7.0 Hz), 7.44 (2H, d, J=8.2 Hz), 7.67 (1H, NH), 7.85 (1H, NH), 7.99 (2H, d, J=8.2 Hz), 8.27 (1H, s), 12.5 (1H, OH).

2-[4-[4-(*N*-Methylcarbamoyl)thiazol-2-yl]phenyl]propionic Acid (23) This compound was prepared in a similar way as in the synthesis of **22**, with use of methylamine to give **23** (48% from XIX). IR (KBr): 3500—2300, 1710, 1620 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.41 (3H, d, J=7.2 Hz), 2.84 (3H, d, J=4.8 Hz), 3.77 (1H, q, J=7.2 Hz), 7.44 (2H, d, J=8.4 Hz), 8.01 (2H, d, J=8.4 Hz), 8.24 (1H, s), 8.46 (1H, q, J=4.8 Hz, NH), 12.4 (1H, OH).

2-[4-[4-(*N*,*N*-Dimethylcarbamoyl)thiazol-2-yl]phenyl]propionic Acid (24) This compound was prepared in a similar way as in the synthesis of **22** with use of dimethylamine to give **24** (49% from XIX). IR (KBr): 3100—2300, 1715, 1590 cm^{-1}. ^{1}H-NMR (DMSO-d_6): 1.41 (3H, d, J=7.2 Hz), 3.03 (3H, s), 3.20 (3H, s), 3.76 (1H, q, J=7.2 Hz), 7.43 (2H, d, J=8.2 Hz), 7.92 (2H, d, J=8.2 Hz), 8.08 (1H, s), 12.4 (1H, OH).

2-[4-(4-Trifluoromethylthiazol-2-yl)phenyl]propionic Acid (25) 1) Diethyl 2-Methyl-2-[4-(4-trifluoromethyl-4-hydroxy-2-thiazolin-2-yl)phenyl]-malonate (XX): The procedure used for preparation of XI-12 was repeated with IV-1 (1.5 g) and 1,1,1-trifluoro-3-bromo-2-propanone (1.2 g) in dry benzene (25 ml) to give XX (1.3 g, 64%). IR (KBr): 3100, 1730, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.26 (6H, t, J=7.1 Hz), 1.68 (3H, s), 3.00 (1H, OH), 3.57 (1H, m), 3.77 (1H, m), 4.25 (4H, q, J=7.1 Hz), 7.45 (2H, d, J=8.7 Hz), 7.90 (2H, d, J=8.7 Hz).

2) 2-[4-(4-Trifluoromethylthiazol-2-yl)phenyl]propionic Acid (**25**): The procedure used for the preparation of **12** was repeated with XX (1.14 g) and 1 N NaOH (9.0 ml) in ethanol (8 ml) to give 2-[4-(4-trifluoromethyl-4-hydroxy-2-thiazolin-2-yl)phenyl]propionic acid (590 mg, 69%). The compound obtained (570 mg) in 12 N HCl (5 ml) was heated at reflux for 170 min. After the reaction, the mixture was extracted with chloroform. The extract was washed with water and brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on silica gel with AcOEt–hexane and recrystallized from AcOEt–hexane to give **25** (410 mg, 75%). IR (KBr): 3500—2600, 1710, 1695 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.55 (3H, d, J=7.2 Hz), 3.80 (1H, q, J=7.2 Hz), 7.42 (2H, d, J=6.7 Hz), 7.72 (1H, m), 7.81 (2H, d, J=6.7 Hz). The compounds **26**, **27**, **36**, **37**, **44**, and **45** were prepared in a similar way as in the synthesis of **1** except that ethanol and acetic acid (1 : 1) were used as solvents for the synthesis of the thiazole ring. The yields and physical properties are summarized in Table IV.

Acknowledgement We thank the staff of the Central Research Laboratory, Green Cross Corporation, for their cooperation in biological and analytical examinations.

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Preparation of Glycyrrhetic Acid Glycosides Having Various β(1→2)-Linked Disaccharides and Their Cytoprotective Effects on Carbon Tetrachloride-Induced Hepatic Injury

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Glycyrrhetic acid glycosides (1—7) having β(1→2)-linked disaccharides such as 2-*O*-β-D-glucopyranosyl-β-D-galactopyranose, 2-*O*-β-D-galactopyranosyl-β-D-galactopyranose, 2-*O*-β-D-glucuronopyranosyl-β-D-galactopyranose, 2-*O*-β-D-glucopyranosyl-β-D-glucuronopyranose, 2-*O*-β-D-galactopyranosyl-β-D-glucuronopyranose, 2-*O*-β-D-galactopyranosyl-β-D-glucopyranose, 2-*O*-β-D-glucuronopyranosyl-β-D-glucopyranose, respectively, were synthesized by stepwise construction; from glycyrrhetic acid monoglycosides to the diglycosides. The cytoprotective activities of the glycosides 1—7 and 2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl-11-oxoolean-12-en-30-oate (8) were compared with natural occurring glycyrrhizin (9). Among these glycosides 1—8, glycosides 3 and 7 having β-D-glucuronopyranose (glcUA) as the only terminal sugar component were more effective materials against hepatic injury than glycyrrhizin 9.

Keywords glycyrrhetic acid glycoside; glycyrrhizin; cytoprotective effect; carbon tetrachloride-induced hepatotoxicity; alanine transaminase activity; aspartate transaminase activity

Naturally occurring saponins (steroidal and triterpenoidal glycosides) isolated from plant sources have various pharmacological and biological activities.[1,2] The biological activities was presumed to depend not only on the structure of aglycons but on the variety, number, conformation, and linking manner of sugars in the saponin molecules.[2]

In this paper, we will report on the preparation of glycyrrhetic acid β-glycosides (1—7) having various β(1→2)-linked disaccharides made up of a combination of D-glucopyranose, D-galactopyranose and D-glucuronopyranose. The comparison of the cytoprotective effects on carbon tetrachloride (CCl_4)-induced hepatic injury of the synthetic saponins 1—7 and 8[3] with glycyrrhizin (9) are also reported.

It has been reported that the glycosidation of 1-methyl cyclohexanol with β(1→2)-linked disaccharide derivative such as 2-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide (**10**) gave only α-glycoside.[4] The same result was also obtained by the reaction of bromide **10** with methyl glycyrrhetinate (**11**) to give α-glycoside,[3] which was thought that because of the absence of an acetyl group at C-2 of compound **10**, the anomeric carbon of **10** was unsusceptible to neighboring-group participation with the acetyl group. Thus, as it is thought that the glycosidation of **11** with the β(1→2)-linked disaccharides affords undesirable α-glycosides, the strategy of stepwise construction from monosaccharide glycosides to disaccharide glycosides is performed for the preparation of desirable β-glycosides. 2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-D-glucopyranose (**12**) and -D-galactopyranose (**13**) used for the first step of glycosidation with **11** have already been made available.[5,6] Other methyl D-glucuronatopyranose derivatives (**14**) and (**15**) were obtained as follows: Methyl 2,3,4-tri-*O*-acetyl-α-D-glucuronatopyranosyl bromide (**16**)[7] was reacted with tetra-*n*-buthylammonium bromide and ethanol[8] to give 1,2-orthoacetate (**17**) (mixture of *exo*- and *endo*-isomers). Treatment of **17** with ammonia followed by benzylation[5,9] gave benzylated orthoacetate (**18**), which was treated with 95% aqueous acetic acid[10] to afford **14**. Acetylation of **14** gave diacetate (**15**) which showed the presence of two acetyl

	R		R
1	-gal $\frac{2}{}$ glc	6	-glc $\frac{2}{}$ gal
2	-gal $\frac{2}{}$ gal	7	-glc $\frac{2}{}$ glcUA
3	-gal $\frac{2}{}$ glcUA	8	-glc $\frac{2}{}$ glc
4	-glcUA $\frac{2}{}$ glc	9	-glcUA $\frac{2}{}$ glcUA
5	-glcUA $\frac{2}{}$ gal		

11 : $R_1=CH_3$, $R_2=\beta$-H
35 : $R_1=H$, $R_2=\beta$-H
36 : $R_1=H$, $R_2=\alpha$-H

glc : β-D-glucopyranose gal : β-D-galactopyranose
glcUA : β-D-glucuronopyranose

Chart 1

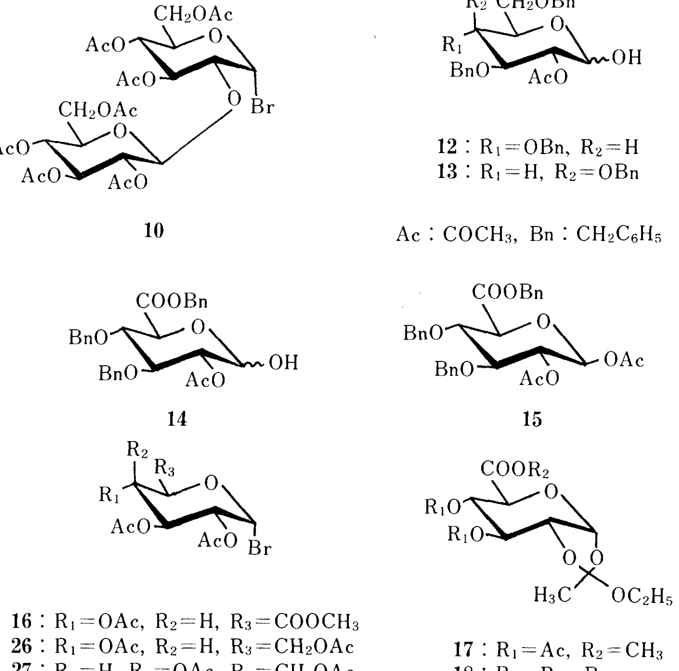

Chart 2

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groups (δ 1.95 and 2.06) and three benzyl groups, and the absence of OCH_3 group in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum.

Glycosidation of aglycon **11** with **13** performed in dichloromethane in the presence of cobalt bromide and trimethylsilyl chloride[11] gave β-glycoside (**19**, 31.5%) and α-glycoside (**20**, 19%). The ^{1}H-NMR spectra of **19** and **20** showed the anomeric protons at δ 4.35 (d, J=8.0 Hz) and 5.19 (d, J=3.7 Hz), respectively. Because of the slight solubility of compound **14** in dichloromethane, the glycosidation of **11** with **15** obtained from **14** was carried out in dichloromethane in the presence of trimethylsilyl trifluoromethanesulfonate[12] to give β-glycoside (**21**, 16%) and α-glycoside (**22**, 29%). The ^{1}H-NMR spectra of **21** and **22** showed the anomeric protons at δ 4.53 (d, J=7.5 Hz) and 5.19 (d, J=3.6 Hz), respectively. Compounds **19** and **21** were treated with 1.5 N NaOMe–MeOH to obtain deacetylated products (**23**) and (**24**), respectively. In the ^{1}H-NMR spectra of **23** and **24**, the signals of acetyl groups (δ 2.02 in **19** and 1.93 in **21**) disappeared.

These glycosides **23** and **24** and the reported one **25**[3] were further reacted with acetylated sugar bromide **26**, **27** or **16** to produce the glycosides having various $\beta(1\rightarrow2)$-linked disaccharides. The reaction of **23** with bromide **26** in dichloromethane in the presence of silver trifluoromethanesulfonate and 1,1,3,3-tetramethyl urea[13] gave a mixture which was chromatographed to obtain a major product (85%) showing a single spot on thin layer chromatography (TLC) (benzene–acetone, 9 : 1; benzene–AcOEt, 4 : 1). The ^{1}H-NMR spectrum of the product, however, showed that it was still contaminated with a small amount of impurity. The pure compound **28** was isolated in the yield of 53% by preparative high-performance liquid chromatography (HPLC) (solvent c). The ^{1}H-NMR spectrum of **28** (Table I) showed the two anomeric protons at δ 4.30 (d, J=7.7 Hz, one of the inner glycosidic linkage) and 5.10 (d, J=8.1 Hz, another of the terminal glycosidic linkage), which suggests that both sugar molecules in **28** arrange in β-configuration. The glycosidations of **23** with **27** and **16** under the same reaction condition gave, after purification by column chromatography followed by preparative HPLC, products (**29**) and (**30**), respectively. Similarly the glycosidation of **24** with **26** and **27** gave products (**31**) and (**32**), respectively, and that of **25** with **27** and **16** afforded products (**33**) and (**34**), respectively. In the ^{1}H-NMR spectra of **29**—**34** (Table I), all anomeric protons showed the coupling constants of 7.7—8.1 Hz, which indicated that both sugar molecules in the products arranged in β-configuration as well as those in **28**. Removal of the protecting groups of compound **28** was performed successively by treatment with NaOMe in MeOH, hydrogenation, and treatment 5% NaOH in H_2O–EtOH (1 : 1). The resulting reaction mixture was chromatographed on silica gel to give the product. Although the product showed a clean single spot on TLC ($CHCl_3$–MeOH–H_2O, 65 : 35 : 10, lower layer), two peaks appeared at the retention times of 52.4 and 56.7 min on HPLC (ODS-1251-D, solvent e, flow rate 0.2 ml/min, 35 °C). The major product **1**, having a retention time of 52.4 min on HPLC, was isolated by preparative HPLC. Similarly, the removal of the protecting groups of **29**—**34** gave, after purification by column chromatography followed by preparative HPLC, products (**2**—**7**), respectively.

Beaton and Spring[14] reported that the reaction of 18β-glycyrrhetic acid (**35**) with alkali and strong acid gave 18α-glycyrrhetic acid (**36**). In order to elucidate the configuration of C-18 on the aglycons, glycosides **1**—**7** were methanolyzed and treated with diazomethane. The all resulting methylated aglycons were identified with authentic **11** by analytical HPLC. This result suggests that the aglycons of **1**—**7** are 18β-glycyrrhetic acid. The ^{13}C-NMR spectra of **1**—**7** are listed together with those of **8** and **9** in Table II.

The cytoprotective effects on CCl_4-induced hepatotoxicity with glycyrrhetic acid glycosides **1**—**9** were estimated by assay of aspartate transaminase (AST) and alanine transaminase (ALT) which were released from CCl_4-treated

19 : R_1=H, R_2=OBn, R_3=CH_2OBn, R_4=Ac
21 : R_1=OBn, R_2=H, R_3=COOBn, R_4=Ac
23 : R_1=H, R_2=OBn, R_3=CH_2OBn, R_4=H
24 : R_1=OBn, R_2=H, R_3=COOBn, R_4=H
25 : R_1=OBn, R_2=H, R_3=CH_2OBn, R_4=H

20 : R_1=H, R_2=OBn, R_3=CH_2OBn
22 : R_1=OBn, R_2=H, R_3=COOBn

Chart 3

28 : R_1=R_5=H, R_2=OBn, R_3=CH_2OBn, R_4=OAc, R_6=CH_2OAc
29 : R_1=R_4=H, R_2=OBn, R_3=CH_2OBn, R_5=OAc, R_6=CH_2OAc
30 : R_1=R_5=H, R_2=OBn, R_3=CH_2OBn, R_4=OAc, R_6=$COOCH_3$
31 : R_1=OBn, R_2=R_5=H, R_3=COOBn, R_4=OAc, R_6=CH_2OAc
32 : R_1=OBn, R_2=R_4=H, R_3=COOBn, R_5=OAc, R_6=CH_2OAc
33 : R_1=OBn, R_2=R_4=H, R_3=CH_2OBn, R_5=OAc, R_6=CH_2OAc
34 : R_1=OBn, R_2=R_5=H, R_3=CH_2OBn, R_4=OAc, R_6=$COOCH_3$

Chart 4

TABLE I. ^{1}H-NMR Spectra of Compounds **28**—**34**[a)]

	28	**29**	**30**	**31**
CH_3	0.81, 0.83, 1.08, 1.12, 1.13, 1.15, 1.34	0.86, 1.11, 1.12, 1.13, 1.18, 1.20, 1.32	0.80, 0.83, 1.05, 1.11, 1.13, 1.15, 1.34	0.80, 0.81, 1.07, 1.10, 1.12, 1.15, 1.36
OCH_3	3.69	3.66	3.69, 3.70	3.69
Aromatic protons	7.24—7.43 (15H)	7.15—7.35 (15H)	7.23—7.45 (15H)	6.79 (1H), 7.22—7.41 (14H)
$-CH_2-$	4.37 (2H, s)	4.49 and 4.75 (2H, ABq, 12.1)	4.38 (2H, s)	4.42 and 4.46 (2H, ABq, 13.9)
	4.51 and 4.80 (2H, ABq, 12.1)	4.56 and 4.99 (2H, ABq, 10.3)	4.58 (2H, s)	4.56 and 4.70 (2H, ABq, 10.3)
	4.57 and 4.63 (2H, ABq, 11.0)	4.71 and 4.75 (2H, ABq, 5.9)	4.51 and 4.79 (2H, ABq, 11.7)	4.62 and 4.85 (2H, ABq, 10.3)
H-3	3.07 (dd, 10.3, 6.2)	3.06 (dd, 10.4, 6.3)	3.06 (dd, 8.3, 6.2)	3.08 (dd, 9.1, 6.3)
H-9	2.27 (s)	2.28 (s)	2.29 (s)	2.31 (s)
H-12	5.66 (s)	5.66 (s)	5.66 (s)	5.68 (s)
H-18	2.75 (br d, 13.2)	2.77 (br d, 13.2)	2.65 (br d, 13.1)	2.80 (br d, 13.1)
H-1′	4.30 (d, 7.7)	4.31 (d, 7.7)	4.30 (d, 7.7)	4.47 (d, 7.7)
H-2′	4.08 (dd, 9.9, 7.7)	3.77 (dd, 8.5, 7.7)	4.08 (dd, 10.3, 7.7)	3.77 (dd, 9.2, 7.7)
H-3′	3.45 (dd, 9.9, 2.7)	{3.5—3.6	3.44 (dd, 10.3, 2.9)	{3.57—3.66
H-4′	3.76 (d, 2.7)		3.77 (d, 2.9)	
H-5′		3.40 (m)		3.97 (d, 9.5)
H-6′$_a$	{3.42—3.52	{3.5—3.6	{3.43—3.52	—
H-6′$_b$				—
H-1″	5.10 (d, 8.1)	5.00 (d, 7.7)	5.11 (d, 8.1)	4.93 (d, 7.7)
H-2″	5.00 (dd, 8.4, 8.1)	5.18 (dd, 10.6, 7.7)	5.01 (dd, 9.8, 8.1)	4.97 (dd, 8.3, 7.7)
H-3″	5.17 (t, 8.4)	4.92 (dd, 10.6, 3.3)	5.17 (t, 9.0)	5.07 (t, 8.3)
H-4″	5.18 (t, 8.4)	5.30 (d, 3.3)	5.23 (t, 9.0)	5.09 (t, 8.3)
H-5″	3.69 (m)	3.77 (m)	4.00 (d, 9.0)	3.52 (m)
H-6″$_a$	4.05 (dd, 11.5, 2.9)	4.03 (dd, 11.0, 6.5)	—	4.02 (dd, 12.1, 1.3)
H-6″$_b$	4.26 (dd, 11.5, 4.0)	4.08 (dd, 11.0, 6.4)	—	4.22 (dd, 12.1, 3.6)
Ac	1.97, 2.00, 2.01, 2.10	1.95, 2.00, 2.02, 2.09	1.99, 1.99, 2.01	2.00, 2.00, 2.01, 2.03

	32	**33**	**34**
CH_3	0.81, 0.85, 1.12, 1.12, 1.12, 1.15, 1.36	0.81, 0.89, 1.14, 1.14, 1.26, 1.26, 1.35	0.81, 0.86, 1.08, 1.13, 1.15, 1.15, 1.35
OCH_3	3.70	3.70	3.69, 3.69
Aromatic protons	6.78 (1H), 7.22—7.41 (14H)	7.29—7.40 (15H)	7.21—7.41 (15H)
$-CH_2-$	4.40 and 4.51 (2H, ABq, 14.6)	4.58 and 4.86 (2H, ABq, 10.3)	4.53 and 4.59 (2H, ABq, 11.7)
	4.55 and 4.69 (2H, ABq, 10.3)	4.57 (2H, s)	4.59 and 4.88 (2H, ABq, 10.3)
	4.62 and 4.85 (2H, ABq, 10.3)	4.76 and 4.88 (2H, ABq, 10.3)	4.74 and 4.77 (2H, ABq, 11.4)
H-3	3.10 (dd, 10.6, 5.9)	3.11 (dd, 10.5, 6.1)	3.09 (dd, 10.6, 5.9)
H-9	2.32 (s)	2.32 (s)	2.31 (s)
H-12	5.68 (s)	5.68 (s)	5.67 (s)
H-18	2.77 (br d, 13.6)	2.81 (br d, 13.0)	2.80 (br d, 13.6)
H-1′	4.47 (d, 7.7)	4.31 (d, 7.7)	4.34 (d, 7.7)
H-2′	3.76 (dd, 9.5, 7.7)	3.87 (dd, 9.1, 7.7)	3.78 (t, 7.7)
H-3′	{3.57—3.66	{3.51—3.60	{3.50—3.65
H-4′			
H-5′	3.95 (d, 8.4)	3.44 (m)	3.43 (m)
H-6′$_a$	—	{3.51—3.60	{3.50—3.65
H-6′$_b$	—		
H-1″	4.92 (d, 7.7)	5.03 (d, 8.1)	5.09 (d, 7.7)
H-2″	5.17 (dd, 10.3, 7.7)	5.20 (dd, 10.3, 8.1)	4.99 (dd, 8.4, 7.7)
H-3″	4.92 (dd, 10.3, 2.6)	4.95 (dd, 10.3, 3.3)	5.13 (t, 8.4)
H-4″	5.33 (d, 2.6)	5.34 (d, 3.3)	5.23 (dd, 9.6, 8.4)
H-5″	3.76 (m)	3.71 (m)	3.89 (d, 9.6)
H-6″$_a$	4.05 (dd, 11.0, 6.2)	4.07 (dd, 12.4, 5.5)	—
H-6″$_b$	4.11 (dd, 11.0, 7.7)	4.13 (dd, 12.4, 5.5)	—
Ac	1.98, 2.01, 2.02, 2.13	1.99, 2.03, 2.05, 2.14	2.00, 2.01, 2.04

a) Coupling constants are given in hertz (Hz).

hepatocytes. The reaction suspensions for assay were composed of hepatocytes (2×10^6 cells) and glycosides (6.3×10^{-7} M or 12.6×10^{-7} M) in Hanks solution (total 1.1 ml), and the suspension for control (C) composed of only hepatocytes (2×10^6 cells) in Hanks solution (total 1.1 ml). The suspensions were exposed to vapor of CCl_4 at 37 °C for 1 h. After incubation of the cell suspensions, the supernatants were collected by centrifugation at $1000 \times g$ for 30 s. The activities of AST and ALT were assayed by the reported procedure.[15)] In the case of lower concentration (Fig. 1), glycosides **3**, **4**, **5**, **7** and **9** having one or two β-D-glucuronopyranoses (glcUA) in the glycoside molecules decreased the releasing of both AST and ALT from hepatocytes injured with CCl_4, which showed a cytoprotective effect. However, glycosides **1**, **2**, **6** and **8** having no gluUA in the molecules showed no effect. At a higher concentration of glycosides (Fig. 2), glycosides **4** and **9** which have gluUA as the inner sugar component released much

TABLE II. ^{13}C-NMR Chemical Shifts of Saponins **1**—**9** in C_5D_5N[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
C-3	88.5	88.6	88.7	88.9	86.1	88.5	88.9	88.7	88.7
C-5	55.3	55.4	55.4	55.3	55.2	55.3	55.4	55.3	53.6
C-9	61.9	60.9	61.9[b]	62.0	62.0	61.0	62.0	60.8	60.4
C-11	199.4	199.4	199.3	199.3	199.4	199.2	199.5	199.2	200.2
C-12	128.5	128.5	128.5	128.5	128.6	128.4	128.6	128.5	126.4
C-13	169.5	169.5	169.3	169.5	169.4	169.2	169.6	169.9	170.5
C-30	179.0	180.8	178.9	179.0	179.0	179.4	179.1	180.7	179.6
C-1′	105.1	104.9	105.0	105.1	105.3	104.6	104.7	104.7	101.5
C-2′	81.9	84.1	83.4	82.7	82.4	83.8	84.3	83.1	77.2
C-3′	76.1	77.6	78.0	76.9	76.5[b]	77.8[b]	78.1[b]	77.8[b]	75.0
C-4′	69.7	71.3	69.6	72.9	72.2	71.1	71.3	71.2[c]	71.2[b]
C-5′	76.7	78.0	77.5	78.1	78.4	77.3[b]	78.0[b]	77.7[b]	74.3
C-6′	62.0[b]	62.6	62.0[b]	172.2	172.0	62.4	62.6	62.0	174.4
C-1″	105.1	106.9	106.7	105.9	106.2	106.6	106.3	105.7	102.5
C-2″	75.1	74.5[b]	76.1	77.2	74.4	74.2[c]	76.5	76.7	73.7
C-3″	77.7	74.7[b]	76.4	77.7[b]	74.9	74.4[c]	77.5	77.9[b]	75.8
C-4″	71.4	69.3	72.9	71.5	70.1	69.1	73.2	71.4[c]	71.1[b]
C-5″	77.7	76.6	75.2	77.5[b]	76.8[b]	76.4	77.9[b]	77.5[b]	75.6
C-6″	62.5[b]	61.1	171.8	62.6	61.2	61.9	172.6	62.5	174.4

a) Characteristic values for the carbons of aglycons were only listed. *b*, *c*) These values may be interchangeable in each column.

TABLE III. Elemental Analyses

Compound	Formula	Calcd (Found)	
		C	H
1	$C_{42}H_{66}O_{14} \cdot H_2O$	62.05 (61.78	8.43 8.49)
2	$C_{42}H_{66}O_{14} \cdot H_2O$	62.05 (61.83	8.43 8.45)
3	$C_{42}H_{64}O_{15} \cdot H_2O$	61.00 (60.58	8.04 7.97)
4	$C_{42}H_{64}O_{15} \cdot H_2O$	61.00 (60.77	8.04 8.13)
5	$C_{42}H_{64}O_{15} \cdot H_2O$	61.00 (60.95	8.04 8.06)
6	$C_{42}H_{66}O_{14} \cdot 1/2H_2O$	62.74 (62.75	8.40 8.47)
7	$C_{42}H_{64}O_{15} \cdot H_2O$	61.00 (60.84	8.04 8.21)

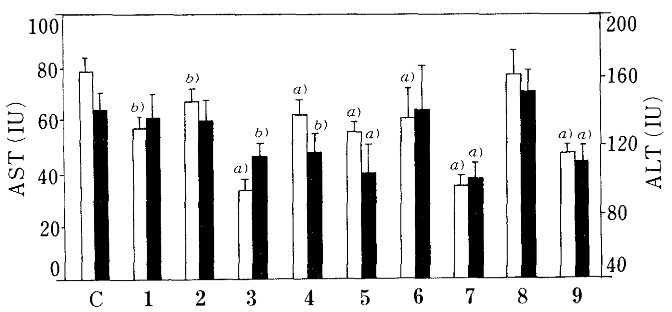

Fig. 1. Cytoprotective Effects of Low Concentration of Glycyrrhetic Acid Glycosides on CCl_4-Induced Hepatic Injury

A final concentration at 6.3×10^{-7} M of glycosides was added to an incubation mixture. Both AST and ALT were assayed as described in the text. Blank bars indicate activities of ALT and closed ones those of AST. Significantly different from the control: *a*) $p<0.01$, *b*) $p<0.05$.

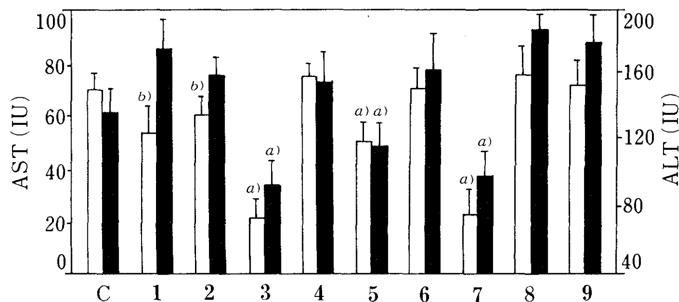

Fig. 2. Cytoprotective Effects of High Concentration of Glycyrrhetic Acid Glycosides on CCl_4-Induced Hepatic Injury

A final concentration at 12.6×10^{-7} M of glycosides was added to an incubation mixture. Both AST and ALT were assayed as described in the text. Blank bars indicate activities ALT and closed ones those of AST. Significantly different from the control: *a*) $p<0.01$, *b*) $p<0.05$.

Fig. 3. Feasible Conformation of Compound **9**

more AST and ALT than glycosides **1**, **2**, **6** and **8**, indicating acceleration of hepatic injury. On the other hand, glycosides **3** and **7** having glcUA as the terminal sugar component indicated strong cytoprotective effects on the CCl_4-injury of hepatocytes. These data suggest that glcUA is essential for cytoprotective effect and the terminal glcUA is more important than the inner one.

The feasible conformation of **9** is illustrated in Fig. 3. Figure 4 shows the proposal fitting model between active sites on the cell and glycyrrhetic acid glycosides having glcUA in the molecules. The terminal glcUA molecule must be bound to the recognition site and aglycon moiety just fixed to the cell surface of hepatocytes, resulting in the inhibition of releasing of inner enzymes (Fig. 4a). If the inner glcUA molecule is bound to the recognition site, the aglycon moiety does not become fixed to the cell surface so that the cytoprotective effect becomes weaker or the membrane is

Fig. 4. A Proposal Model of Effects of Glycyrrhetic Acid Glycosides on Hepatocytes

destroyed (Fig. 4b).

Experimental

Materials Methyl glycyrrhetinate was prepared by methylation of glycyrrhetic acid according to the published procedure.[16] Dry dichloromethane (CH_2Cl_2) was obtained by refluxing with NaH followed by distillation. Other chemicals and solvents were of reagent grade, and were obtained from commercial sources.

Measurements The TLC utilized Kieselgel HF_{254} (Merck), and spots were detected by irradiating the plates with ultraviolet (UV) light (254 nm, Manasul-light) and spraying with dilute H_2SO_4 followed by heating at 80 °C for 10 min. Column chromatography was carried out on Wakogel C-200. An SSC-6300 (Senshu Scientific Co., Ltd.) equipped with an SSC-3000 A was employed for analytical HPLC using ODS-1251-D (4.6 mm × 250 mm), and was further equipped with an SSC autoinjector 6310 and an SSC fraction collector 6320 for preparative HPLC using ODS-4521-D (10 mm × 250 mm). The following solvent systems for HPLC were used: (a) 20% H_2O–acetone, (b) 22.5% H_2O–acetone, (c) 25% H_2O–acetone, (d) MeOH–H_2O–AcOH (64.5:35:0.5), and (e) MeOH–H_2O–AcOH (69.5:35:0.5). Optical rotations were measured with a JASCO J-20A spectropolarimeter. ^1H- and ^{13}C-NMR spectra were obtained with a JEOL JNM-GX NMR spectrometer at 270 and 67.8 MHz, respectively, and chemical shifts were given in ppm with tetramethylsilane as an internal standard. Electron impact and fast atom bombardment mass spectra (EI- and FAB-MS) were recorded on a JEOL JMS-DX 300 mass spectrometer. The activities of AST and ALT were assayed by autoanalyzer COBAS MRA (Roche) using commercial kits based on the principal of the AST and ALT assay method.[15]

Preparation of Hepatocytes Isolated rat hepatocytes were prepared using 150—180 g Wistar rats by a slightly modified method of Seglen.[17]

Methyl 3,4-Di-*O*-acetyl-α-D-glucuronatopyranose 1,2-*O*-(Ethyl Orthoacetate) 17 To a solution of methyl 2,3,4-tri-*O*-acetyl-α-D-glucuronatopyranosyl bromide **16** (100 g) in γ-collidine (200 ml), tetra-*n*-butylammonium bromide (20 g) and dry ethanol (14 ml) were added, and the mixture was stirred for 24 h at 50 °C. After filtration, the filtrate was poured into ice-water (500 ml) and extracted with CH_2Cl_2 (300 ml × 3). The combined extracts were washed successively with 5% aqueous HCl, aqueous-saturated $NaHCO_3$, and water, then dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography to obtain **17** (50 g, 55%). The ^1H-NMR spectrum of **17** indicated the formation of *exo*- and *endo*-1,2-orthoacetate (5:1), which were not isolated. ^1H-NMR spectrum of the *exo*-1,2-orthoacetate ($CDCl_3$) δ: 5.86 (1H, d, J=4.8 Hz, H-1), 5.24 (1H, dd, J=2.9, 2.2 Hz, H-3), 5.15 (1H, ddd, J=7.3, 2.2, 1.1 Hz, H-4), 4.32 (1H, ddd, J=4.8, 2.9, 1.1 Hz, H-2), 4.31 (1H, d, J=7.3 Hz, H-5), 3.78 (3H, s, OCH_3), 3.55 (2H, q, J=7.0 Hz, –$\underline{CH_2}$–CH_3), 2.11 and 2.10 (each 3H, Ac), 1.75 (3H, s, CH_3), 1.18 (3H, t, J=7.0 Hz, –CH_2–$\underline{CH_3}$). EI-MS *m/z* (%): 317 (9, $M^+ - OCH_2CH_3$), 303 (8, M^+ – OAc), 275 (11), 257 (11), 231 (5), 215 (15), 214 (15), 197 (12), 186 (6), 173 (24), 172 (15), 157 (15), 156 (11), 155 (100). *Anal.* Calcd for $C_{15}H_{22}O_{10}$: C, 49.72; H, 6.12. Found: C, 49.65; H, 6.16.

Benzyl 3,4-Di-*O*-benzyl-α-D-glucuronatopyranose 1,2-*O*-(Ethyl Orthoacetate) 18 To a solution of **17** (45 g) in MeOH (300 ml), ammonia-saturated MeOH (70 ml) was added, and the mixture was allowed to stand for 20 h at room temperature. After removing ammonia by aspiration, the solution was evaporated to give a residue (38 g). The residue was dissolved in dimethylformamide (100 ml), added with benzyl bromide (50 ml) and silver oxide (35 g), and stirred in the dark for 20 h at room temperature. The reaction mixture was added with MeOH (30 ml), and stirred for another 2 h. The mixture was filtered through Kieselguhr. The filtrate was concentrated to give a residue which was subjected to column chromatography (toluene–AcOEt, 4:1) to obtain **18** (15.3 g, 23%). *Anal.* Calcd for $C_{31}H_{34}O_8$: C, 69.65; H, 6.41. Found: C, 70.07; H, 6.53.

Benzyl 2-*O*-Acetyl-3,4-di-*O*-benzyl-D-glucuronatopyranose 14 The foregoing 1,2-orthoester **18** (12 g) was dissolved in 95% aqueous acetic acid (200 ml), and the mixture was allowed to stand for 15 min at room temperature. The mixture was poured into ice-water (50 ml) and extracted with CH_2Cl_2 (50 ml × 3). The combined organic extracts were washed successively with aqueous-saturated $NaHCO_3$ and water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was purified by column chromatography (benzene–acetone, gradient up to 5%) to yield pure **14** (9.4 g, 83%). This product showed a singlet peak at δ: 2.02 due to an acetyl group in the ^1H-NMR spectrum. FAB-MS *m/z*: 506 (M^+). *Anal.* Calcd for $C_{29}H_{30}O_8$: C, 68.76; H, 5.97. Found: C, 68.39; H, 6.10.

Benzyl 1,2-Di-*O*-acetyl-3,4-di-*O*-benzyl-β-D-glucuronatopyranose 15 Compound **14** (6.4 g) was dissolved in acetic anhydride (5 ml) and pyridine (5 ml), and the mixture was allowed to stand overnight at room temperature. The mixture was concentrated to give a residue (6.7 g) which was subjected to column chromatography (benzene–acetone, gradient up to 10%) to obtain **15** (3.3 g, 48%). ^1H-NMR ($CDCl_3$) δ: 7.36—7.21 (15H, aromatic protons), 5.78 (1H, d, J=7.3 Hz, H-1), 5.06 (1H, t, J=7.3 Hz, H-2), 4.76—4.40 (6H, –CH_2– × 3 on benzyl groups), 4.20 (1H, d, J=7.0 Hz, H-5), 3.95 (1H, t, J=7.3 Hz, H-3), 3.71 (1H, dd, J=7.3, 7.0 Hz, H-4), 2.06 and 1.95 (each 3H, s, Ac). FAB-MS *m/z*: 548 (M^+). *Anal.* Calcd for $C_{31}H_{32}O_9$: C, 67.87; H, 5.88. Found: C, 67.75; H, 5.89.

Glycosidation of Methyl Glycyrrhetinate 11 with 13 To a solution of **11** (3 g) and 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-D-galactopyranose **13** (5 g) in dry CH_2Cl_2 (10 ml), anhydrous cobalt bromide (2 g), trimethylsilyl chloride (1.3 ml) and molecular sieve (4Å, 1 g) were added. After stirring for 20 h at room temperature, the mixture was centrifuged to give a solution. The solution was washed successively with aqueous $NaHCO_3$ and water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography (benzene–acetone, gradient up to 10%) to obtain compound **19** (1.9 g, 32%) and **20** (1.2 g, 19%). ^1H-NMR of **19** ($CDCl_3$) δ: 0.76, 0.80, 0.90, 1.11, 1.11, 1.14, 1.33 (each 3H, s, CH_3), 2.02 (3H, s, Ac), 2.28 (1H, s, H-9), 2.75 (1H, br d, J=12.6 Hz, H-18), 3.02 (1H, br t, J=8.0 Hz, H-3), 3.48 (1H, dd, J=9.9, 2.7 Hz, H-3'), 3.52—3.63 (3H, H-5', 6a', 6b'), 3.69 (3H, s, OCH_3), 3.91 (1H, d, J=2.7 Hz, H-4'), 4.35 (1H, d, J=8.0 Hz, H-1'), 4.43—4.93 (6H, –CH_2– × 3 on benzyl groups), 5.38 (1H, dd, J=9.9, 8.0 Hz, H-2'), 5.65 (1H, s, H-12), 7.23—7.36 (15H, aromatic protons). FAB-MS *m/z*: 981 $[M+Na]^+$. *Anal.* Calcd for $C_{60}H_{78}O_{10}$: C, 75.12; H, 8.20. Found: C, 75.05; H, 8.26. ^1H-NMR of **20** ($CDCl_3$) δ: 0.80, 0.81, 0.98, 1.12, 1.45, 1.57, 1.57 (each 3H, s, CH_3), 2.05 (3H, s, Ac), 2.30 (1H, s, H-9), 2.75 (1H, br d, J=12.6 Hz, H-18), 3.11 (1H, br d, J=8.0 Hz, H-3), 3.51 (1H, dd, J=8.8, 5.5 Hz, H-6a'), 3.59 (1H, dd, J=8.8, 7.3 Hz, H-6b'), 3.92 (1H, dd, J=10.3, 3.6 Hz, H-3'), 4.01 (1H, d, J=3.6 Hz, H-4'), 4.11 (1H, br t, J=8.8 Hz, H-5'), 4.38—4.96 (6H, –CH_2– × 3 on benzyl groups), 5.19 (1H, d, J=3.7 Hz, H-1'), 5.25 (dd, J=10.3, 3.7 Hz, H-2'), 5.66 (1H, s, H-12), 7.26—7.36 (15H, aromatic protons). FAB-MS *m/z*: 981 $[M+Na]^+$. *Anal.* Calcd for $C_{60}H_{78}O_{10}$: C, 75.12; H, 8.20. Found: C, 75.33; C, 8.15.

Deacetylation of 19 To a solution of compound **19** (1.7 g) in MeOH (40 ml), 1.5 N NaOMe–MeOH (8 ml) was added. After stirring for 20 h at room temperature, the mixture was poured into ice-water (100 ml) and extracted with CH_2Cl_2 (100 ml × 3). The combined organic extracts were washed with H_2O, dried over Na_2SO_4 and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography (benzene–acetone, gradient up to 5%) to afford compound **23** (1.4 g, 86%). ^1H-NMR ($CDCl_3$) δ: 0.80, 0.84, 1.02, 1.11, 1.13, 1.15, 1.34 (each 3H, s, CH_3), 2.30 (1H, s, H-9), 2.76 (1H, br d, J=10.3 Hz, H-18), 3.13 (1H, dd, J=8.8, 4.1 Hz, H-3), 3.43 (1H, dd, J=9.9, 2.9 Hz, H-3'), 3.57 (3H, m, H-5', 6a', 6b'), 3.69 (3H, s, OCH_3), 3.89 (1H, d, J=2.6 Hz, H-4'), 3.98 (1H, dd, J=9.9, 7.7 Hz, H-2'), 4.28 (1H, d, J=7.7 Hz, H-1'), 4.40—4.89 (6H, –CH_2– × 3 on benzyl groups), 5.66 (1H, s, H-12), 7.26—7.37 (15H, aromatic protons). FAB-MS *m/z*: 939 $[M+Na]^+$. *Anal.* Calcd for $C_{58}H_{76}O_9$: C, 75.95; H, 8.35. Found: C, 75.57; H, 8.40.

Glycosidation of 11 with 15 The solution of **11** (1 g) and **15** (2.2 g) in

dry CH_2Cl_2 (20 ml) was added with trimethylsilyl trifluoromethanesulfonate (0.4 ml) and molecular sieve (4 Å, 600 mg). After stirring for 2 d at 50 °C, the mixture was centrifuged to give a supernant which was poured into ice-water (100 ml) and extracted with CH_2Cl_2 (80 ml × 3). The combined organic extracts were washed successively with aqueous-saturated $NaHCO_3$ and water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography (benzene–acetone, gradient up to 5%) to afford compound **21** (320 mg, 16%) and **22** (580 mg, 29%). ^{1}H-NMR spectrum of **21** ($CDCl_3$) δ: 0.73, 0.80, 0.90, 0.91, 1.10, 1.14, 1.35 (each 3H, s, CH_3), 1.93 (3H, s, Ac), 2.30 (1H, s, H-9), 2.77 (1H, br d, J = 10.3 Hz, H-18), 3.06 (1H, dd, J = 8.8, 4.1 Hz, H-3), 3.65 (1H, dd, J = 7.5, 7.3 Hz, H-3′), 3.72 (1H, t, J = 7.3 Hz, H-4′), 4.00 (1H, d, J = 7.3 Hz, H-5′), 4.53 (1H, d, J = 7.5 Hz, H-1′), 4.35—4.83 (6H, $-CH_2-$ × 3 on benzyl groups), 5.00 (1H, t, J = 7.5 Hz, H-2′), 5.68 (1H, s, H-12), 7.22—7.33 (15H, aromatic protons). FAB-MS m/z: 995 $[M+Na]^+$. *Anal.* Calcd for $C_{60}H_{76}O_{11}$: C, 74.04; H, 7.87. Found: C, 73.89; H, 8.01. ^{1}H-NMR spectrum of **22** ($CDCl_3$) δ; 0.80, 0.84, 0.90, 0.95, 1.00, 1.11, 1.14 (each 3H, s, CH_3), 2.00 (3H, s, Ac), 2.30 (1H, s, H-9), 2.69 (1H, br d, J = 10.3 Hz, H-18), 3.14 (1H, br t, J = 7.3 Hz, H-3), 3.36 (1H, H-4′, overlapped with OCH_3), 3.98 (1H, t, J = 8.1 Hz, H-3′), 4.33 (1H, d, J = 9.9 Hz, H-5′), 4.46—4.86 (6H, $-CH_2-$ × 3 on benzyl groups), 4.76 (1H, dd, J = 8.1, 3.6 Hz, H-2′), 5.19 (1H, d, J = 3.6 Hz, H-1′), 5.66 (1H, s, H-12), 7.25—7.34 (15H, aromatic protons). FAB-MS m/z: 995 $[M+Na]^+$. *Anal.* Calcd for $C_{60}H_{76}O_{11}$: C, 74.04; H, 7.87. Found: C, 73.95; H, 7.90.

Deacetylation of 21 To a solution of **21** (200 mg) in MeOH (6 ml), 1.5 N NaOH–MeOH (1.1 ml) was added, and the mixture was allowed to stand for 20 h at room temperature. The mixture was poured into ice-water (20 ml) and extracted with CH_2Cl_2 (20 ml × 3). The combined organic extracts were washed with water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was purified by column chromatography (benzene–acetone, gradient up to 10%) to afford compound **24** (165 mg, 86%). ^{1}H-NMR ($CDCl_3$) δ: 0.81, 0.81, 1.01, 1.10, 1.11, 1.15, 1.36 (each 3H, s, CH_3), 2.37 (1H, s, H-9), 2.77 (1H, br d, J = 13.6 Hz, H-18), 3.14 (1H, t, J = 8.4 Hz, H-3), 3.57—3.65 (3H, H-2′, 3′, 4′), 3.68 (3H, s, OCH_3), 3.96 (1H, d, J = 8.4 Hz, H-5′), 4.43 (1H, d, J = 7.7 Hz, H-1′), 4.61—4.82 (6H, $-CH_2-$ × 3 on benzyl groups), 5.68 (1H, s, H-12), 6.80 (1H), 7.25—7.40 (14H) (aromatic protons). FAB-MS m/z: 953 $[M+Na]^+$. *Anal.* Calcd for $C_{58}H_{74}O_{10}$: C, 74.81; H, 8.01. Found: C, 74.77; H, 8.07.

Glycosidation of 23 with 26 To a solution of **23** (700 mg) and **26** (1 g) in dry CH_2Cl_2 (15 ml), silver trifluoromethanesulfonate (AgOTf) (700 mg) and 1,1,3,3-tetramethylurea (TMU) (0.5 ml) was added, and the mixture was stirred for 4 h at room temperature. The mixture was centrifuged, and the supernant was poured into ice-water (50 ml) and extracted with CH_2Cl_2 (50 ml × 3). The combined organic extracts were washed successively with aqueous-saturated $NaHCO_3$ and H_2O, and dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was subjected to column chromatography (benzene–AcOEt, gradient up to 5%) to obtain a major product (795 mg, 85%). The product was further purified by preparative HPLC (solvent c) to afford pure compound **28** (495 mg, 53%). FAB-MS m/z: 1285 $[M+K]^+$. *Anal.* Calcd for $C_{72}H_{94}O_{18}$: C, 69.32; H, 7.60. Found: C, 69.30; H, 7.63.

Glycosidation of 23 with 27 The mixture of **23** (1 g), **27** (1.7 g), AgOTf (1 g), TMU (0.83 ml), and dry CH_2Cl_2 (20 ml) was stirred for 4 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue. Pure compound **29** (450 mg, 33%) was isolated by column chromatography (benzene–AcOEt, gradient up to 5%) followed by preparative HPLC (solvent b). FAB-MS m/z: 1285 $[M+K]^+$. *Anal.* Calcd for $C_{72}H_{94}O_{18}$: C, 69.32; H, 7.60. Found; C, 69.05; H, 7.69.

Glycosidation of 23 with 16 The mixture of **23** (500 mg), **16** (1.25 g), AgOTf (800 mg), TMU (0.56 ml) and dry CH_2Cl_2 (10 ml) was stirred for 4 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue which was purified by column chromatography (benzene–AcOEt, gradient up to 5%) followed by preparative HPLC (solvent b) to obtain compound **30** (360 mg, 54%). FAB-MS m/z: 1271 $[M+K]^+$. *Anal.* Calcd for $C_{71}H_{92}O_{18}$: C, 69.13; H, 7.52. Found: C, 69.10; H, 7.52.

Glycosidation of 25 with 27 The mixture of **25** (650 mg), **27** (1.2 g), AgOTf (710 mg), TMU (0.56 ml), and dry CH_2Cl_2 (15 ml) was stirred for 4 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue which was subjected to column chromatography to obtain a crude product (625 mg). The preparative HPLC (solvent c) of the product gave pure compound **33** (240 mg, 27%). FAB-MS m/z: 1285 $[M+K]^+$. *Anal.* Calcd for $C_{72}H_{94}O_{18}$: C, 69.32; H, 7.60. Found: C, 69.57; H, 7.73.

Glycosidation of 25 with 16 The mixture of **25** (500 mg), **16** (2 g), AgOTf (800 mg), TMU (0.56 ml), and dry CH_2Cl_2 (10 ml) was stirred for 5 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue which was subjected to column chromatography (benzene–AcOEt, gradient up to 5%) to obtain crude product **34** (380 mg). The preparative HPLC (solvent a) of the product gave pure compound **34** (260 mg, 39%). FAB-MS m/z: 1271 $[M+K]^+$. *Anal.* Calcd for $C_{71}H_{92}O_{18}$: C, 69.13; H, 7.52. Found: C, 68.89; H, 7.58.

Glycosidation of 24 with 26 The mixture of **24** (150 mg), **26** (300 mg), AgOTf (96 mg), TMU (0.1 ml), and dry CH_2Cl_2 (5 ml) was stirred for 8 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue which was subjected to column chromatography to obtain crude product **31** (150 mg). The preparative HPLC (solvent c) of the product gave pure **31** (100 mg, 49%). FAB-MS m/z: 1299 $[M+K]^+$. *Anal.* Calcd for $C_{72}H_{94}O_{18}$: C, 68.55; H, 7.35. Found: C, 68.35; H, 7.41.

Glycosidation of 24 with 27 The mixture of **24** (260 mg), **27** (350 mg), AgOTf (190 mg), TMU (0.2 ml), and dry CH_2Cl_2 (5 ml) was stirred for 6 h at room temperature. The mixture was post-treated according to the preparation method of **28** to give a residue which was subjected to column chromatography (benzene–AcOEt, gradient up to 5%) to obtain crude product **32** (156 mg). The preparative HPLC (solvent c) gave pure product **32** (110 mg, 31%). FAB-MS m/z: 1299 $[M+K]^+$. *Anal.* Calcd for $C_{72}H_{92}O_{19}$: C, 68.55; H, 7.35. Found: 68.09; H, 7.45.

Removal of the Protecting Groups of 28 To a solution of **28** (610 mg) in MeOH (20 ml), 1.5 N NaOMe–MeOH (4 ml) was added, then the mixture was allowed to stand for 12 h at room temperature. The mixture was neutralized with acetic acid, and evaporated to give a residue which was subjected to column chromatography (MeOH–CH_2Cl_2, 1 : 9) to afford a syrupy product. The product was dissolved in acetic acid (20 ml) and added with 10% Pd–charcoal (30 mg). The mixture was stirred in a hydrogen atmosphere for 20 h at room temperature and filtered. The filtrate was evaporated to give a residue. The residue was dissolved in 5% NaOH in H_2O–EtOH (1 : 1) (6.5 ml) and refluxed for 2 h. After cooling, the mixture was neutralized with acetic acid and evaporated to obtain a residue which was purified by column chromatography ($CHCl_3$–MeOH–H_2O, 65 : 35 : 10) followed by preparative HPLC (solvent e) to yield glycoside **1** (125 mg, 32%). FAB-MS m/z: 817 $[M+Na]^+$. $[\alpha]_D^{25}$ +64.1 (c = 1.56, MeOH).

The Removal of the Protecting Groups of 29—34 The removal of the protecting groups of **29—34** was performed by the same method of **1** to obtain **2** (37%, FAB-MS m/z: 817 $[M+Na]^+$, $[\alpha]_D^{25}$ +53.9 (c = 1.52, MeOH)), **3** (30%, FAB-MS m/z: 831 $[M+Na]^+$, $[\alpha]_D^{25}$ +50.7 (c = 1.40, MeOH)), **4** (42%, FAB-MS m/z: 831 $[M+Na]^+$, $[\alpha]_D^{25}$ +43.6 (c = 1.56, MeOH)), **5** (40%, FAB-MS m/z: 831 $[M+Na]^+$, $[\alpha]_D^{25}$ +55.7 (c = 0.97, MeOH)), **6** (44%, FAB-MS m/z: 817 $[M+Na]^+$, $[\alpha]_D^{25}$ +52.1 (c = 0.96, MeOH)), and **7** (43%, FAB-MS m/z: 831 $[M+Na]^+$, $[\alpha]_D^{25}$ +50.6 (c = 1.42, MeOH)), respectively.

Methanolysis of 1—7 Each 5 mg of sample (**1—7**) was dissolved in 0.5 N HCl–MeOH (1 ml) and refluxed for 2 h. Each reaction mixture was extracted with AcOEt and washed with H_2O, dried over Na_2SO_4, and filtered. The filtrate was evaporated to give a residue which was treated with diazomethane to obtain methylated aglycon. Each aglycon was purified by preparative TLC (benzene–acetone, 85 : 15). All aglycons obtained from **1—7** were identified with authentic **11** by HPLC (retention time: 38.4 min; solvent system, 30% H_2O–acetone; flow rate, 0.2 ml/min).

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Development of Active Center-Directed Inhibitors against Plasmin[1)]

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Active center-directed inhibitors of plasmin were designed based on the structure of specific substrates of plasmin and then synthesized. Their effects on plasmin were examined and the structure–inhibitory activity relationship was studied. N^{α}-*trans*-4-Aminomethylcyclohexanecarbonyllysine 4-benzoylanilide (Tra–Lys–BZA) inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 15 and 6.1 μM, respectively and N^{α}-*trans*-4-aminomethylcyclohexanecarbonyllysine 4-benzylpiperidine amide (Tra–Lys–BPP) did not show any detectable inhibitory activity. Moreover, it was revealed that Tra–Lys–4-methoxycarbonylanilide inhibited plasma kallikrein more potently than plasmin.

Keywords plasmin; competitive inhibitor; design; Lys derivative; synthesis; structure–activity relationship

It is well known that proteinases and their natural inhibitors regulate biological functions cooperatively to maintain homeostasis, and imbalances between proteinases and their natural inhibitors cause serious disorders. With regard to plasmin, an imbalance between plasmin and its natural inhibitors (α_2-macroglobulin, α_2-plasmin inhibitor, *etc.*) also causes serious syndromes, such as hyperfibrinolysis. At present, ε-aminocaproic acid (EACA)[2)] and *trans*-4-aminomethylcyclohexanecarboxylic acid (*trans*-AMCHA)[3)] are employed clinically as plasmin inhibitors. These inhibitors showed fairly potent inhibition of fibrinolysis by plasmin, but very slight inhibition of the amidolysis of small peptide substrates and fibrinogenolysis by plasmin, because these inhibitors exhibit an inhibitory effect on plasmin by blocking the lysine binding site (LBS) of an enzyme, which is not the catalytic site.[4)]

Thus, our research goal was to synthesize active center-directed inhibitors of plasmin, with the objective of obtaining potent and selective inhibitors of plasmin toward not only fibrinolysis but also amidolysis and fibrinogenolysis. This report deals with the design and synthesis of active center-directed plasmin inhibitors.

Previously, we reported that D-Ile–Phe–Lys–*p*NA was a selective substrate with a low K_m value for plasmin,[5)] and that D-Ile–Phe–Lys–BZA inhibited plasmin activities toward S-2251 and fibrin with IC_{50} values of 69 and 180 μM, respectively, by blocking the active center of plasmin, but D-Ile–Phe–Lys–BPP was not inhibitory.[6)] It was also found that a free amino group of D-Ile at the P_3 position[7)] might contribute to an increment in affinity between the substrates or inhibitors and the enzyme.

First of all, we prepared Lys derivatives substituted for the D-Ile–Phe moiety of D-Ile–Phe–Lys–BZA using an amino-containing compound. As shown in Fig. 1, the desired compounds were prepared and their inhibitory activities against plasmin are summarized in Table I. From Table I, it was revealed that the ε-aminocaproyl (Eac) or *trans*-4-aminomethylcyclohexanecarbonyl (Tra) group is suitable for binding with plasmin, presumably because the position of the free amino group of Eac or Tra is similar to that of the free amino group of D-Ile–Phe moiety. The

TABLE I. Inhibitory Effect of R–Lys–NH–C6H4–CO–C6H5 on Plasmin

	R	IC_{50} (μM)		
		S-2251	Fn	Fg
1	$NH_2(CH_2)_4CO$–	16	17	36
2	$NH_2(CH_2)_5CO$–	12	>10 (18%)[a)]	ND
3	NH_2CH_2–(H)–CO– (*trans*)	15	6.1	13
4	NH_2CH_2–(H)–CO– (*cis*)	400	260	ND
5	NH_2–(H)~CO–	>250 (0%)	>250 (6%)	ND
6	(H)~CO– with NH_2	>200 (43%)	64	ND

a) In parentheses, inhibitory % at the concentration described is indicated. ND = not determined.

Boc-Lys(Z)-OH —[H_2N–C6H4–CO–C6H5, $ClCOOC_2H_5$]→ Boc-Lys(Z)-BZA —[HCl/dioxane]→ H-Lys(Z)-BZA·HCl —[Z-Tra-OH, $SOCl_2$]→ Z-Tra-Lys(Z)-BZA —[H_2-Pd]→ Tra-Lys-BZA (**3**)

Fig. 1. Synthetic Route to Tra–Lys–BZA (**3**)

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TABLE II. Inhibitory Effect of NH_2CH_2-⟨H⟩IIICO–R–NH-⟨⟩-CO-⟨⟩ on Plasmin

	R	IC_{50} (μM)		
		S-2251	Fn	Fg
3	Lys	15	6.1	13
7	D-Lys	> 200 (0%)[a]	> 200 (0%)	ND
8	Orn	> 300 (33%)	> 300 (43%)	ND

a) In parentheses, inhibitory % at the concentration described is indicated.

TABLE III. Inhibitory Effect of NH_2CH_2-⟨H⟩IIICO–Lys–R on Plasmin

	R	IC_{50} (μM)	
		S-2251	Fn
9	–N⟨piperidine⟩–$COOC_2H_5$	> 1000 (0%)	> 1000 (19%)[a]
10	–N⟨piperidine⟩–CH_2–⟨⟩	> 500 (7%)	> 500 (16%)
11	–N⟨tetrahydroisoquinoline⟩	> 500 (18%)	> 500 (33%)
12	–NH–⟨⟩–NO_2	> 500 (41%)	530
13	–NH–⟨tetrahydronaphthyl⟩	> 500 (9%)	> 500 (31%)
14	–NH–⟨⟩–$COOCH_3$	> 500 (17%)	330
15	–NH–⟨⟩–$COCH_3$	39	9.3
3	–NH–⟨⟩–CO–⟨⟩	15	6.1
16	–O–⟨⟩–CO–⟨⟩	24	170
17	–CH_2NH–⟨⟩–CO–⟨⟩	450	510
18	–CH_2O–⟨⟩–CO–⟨⟩	660	200

a) In parentheses, inhibitory % at the concentration described is indicated.

compound (**3**) exhibited inhibitory activity against plasmin toward S-2251 and fibrin with IC_{50} values of 15 and 6.1 μM, respectively.

As summarized in Table II, Tra–D-Lys–BZA and Tra–Orn–BZA did not show any detectable inhibitory activity against plasmin, indicating that Lys should be in an L-configuration, and that Orn instead of Lys is not suitable for the manifestation of inhibitory activity.

Next, the BZA moiety of Tra–Lys–BZA (**3**) was substituted by piperidine type compounds and aniline type compounds, and their inhibitory activities against plasmin were examined and the results are summarized in Table III.

Previously, it was reported that Tos–Lys–BPP inhibited plasmin toward S-2251 and fibrin with IC_{50} values of 300 and 150 μM, respectively. However, Tra–Lys–BPP (**10**)

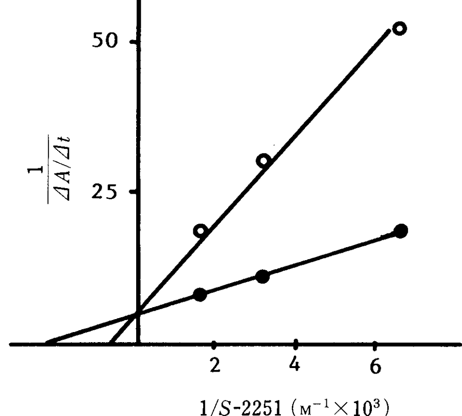

Fig. 2. Mode of Inhibition of Tra–Lys–BZA (**3**) toward Plasmin
○: Tra–Lys–BZA (**3**), ●: no inhibitor.

TABLE IV. Comparison of Inhibitory Activity against Plasmin

Compound	IC_{50} (μM)		
	S-2251	Fn	Fg
NH_2CH_2–⟨H⟩IIIIICOOH (*trans*-AMCHA)	75000	60	9500
CH_3–⟨⟩–SO_2–Lys–NH–⟨⟩–NO_2	700	780	900
CH_3–⟨⟩–SO_2–Lys–N⟨⟩–CH_2–⟨⟩	300	150	ND
CH_3–⟨⟩–SO_2–Lys–NH–⟨⟩–CO–⟨⟩	140	150	200
D-Ile–Phe–Lys–NH–⟨⟩–CO–⟨⟩	69	180	ND
NH_2CH_2–⟨H⟩IIIIICO–Lys–NH–⟨⟩–CO–⟨⟩	15	6.1	13

and other piperidine type derivatives did not show any detectable inhibitory activity against plasmin, as can be seen in Table III. Tra–Lys–piperidine amide derivatives are not suitable for the manifestation of inhibitory activity against plasmin. These facts are compatible with the fact that D-Ile–Phe–Lys–BPP did not inhibit plasmin.[6] Tra–Lys–*p*NA (**12**) weakly inhibited plasmin towards S-2251 and fibrin [IC_{50} = 500 μM (41% inhibition) and 530 μM, respectively], but *p*-nitroaniline was not liberated from Tra–Lys–*p*NA (**12**) by the action of plasmin, indicating that this type of compound is stable to the action of plasmin. Tra–Lys–ACA (**15**) inhibited plasmin activity toward S-2251 and fibrin with IC_{50} values of 39 and 9.3 μM, respectively. Tra–Lys–4-methoxycarbonylanilide (**14**) inhibited plasma kallikrein with an IC_{50} value of 50 μM[8] and plasmin toward S-2251 and fibrin with IC_{50} values of 500 μM (17% inhibition) and 330 μM, respectively, indicating that this compound (**14**) is a plasma kallikrein inhibitor rather than plasmin inhibitor.

Next, the –CONH– bond of Lys–BZA was modified with an ester bond (**16**), aminomethyl ketone, –$COCH_2NH$– (**17**) and oxymethyl ketone, –$COCH_2O$– (**18**). The inhibitory activity of these compounds against plasmin decreased compared with the corresponding amide bond derivative (**3**) as shown in Table III.

As stated above, the Lys–*p*NA bond of Tra–Lys–*p*NA (**12**) was not cleaved by plasmin; however, these com-

pounds, including Tra–Lys–BZA (**3**), inhibited plasmin competitively as shown in Fig. 2. This result posed the question; which amino group (Tra or Lys in **3**) interacts with S_1 position of plasmin[7]? In order to clarify this problem, Z–Tra–Lys–4-benzoylphenoxymethyl ketone (**19**) and Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (**20**), which were derivatives of compound (**18**), were prepared, and their inhibitory activity against plasmin was examined. These derivatives of **18** were selected because the lysyl bond in **18** is stable to the action of plasmin. The fact that Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (**20**) still exhibited weak inhibitory activity against plasmin toward S-2251 and fibrin with IC_{50} values of 200 and 60 μM, respectively, suggested the possibility that the amino group of Tra might interact with the S_1 position of plasmin. Moreover, it was deduced that the existence of an ε-amino group of Lys in compound (**18**) decreased the inhibitory activity of **18** against plasmin from the fact that compound (**20**) exhibited more potent inhibitory activity than the parent molecule (**18**). It is surprising that Tra–Lys derivatives obtained here interact with plasmin in quite a different manner from D-Ile–Phe–Lys derivatives, although Tra–Lys derivatives were designed based on the structure of D-Ile–Phe–Lys derivatives.

Finally, the inhibitory activities obtained above are summarized in Table IV and compaired with the activity of other plasmin inhibitors. As can be seen in Table IV, Lys-derivatives (Tos–Lys–*p*NA, Tos–Lys–BPP, Tos–Lys–BZA *etc.*) exhibited more potent inhibitory activity against plasmin toward S-2251 and fibrinogenolysis. Finally, Tra–Lys–BZA (**3**) inhibited plasmin not only toward S-2251 and fibrinogenolysis but also toward fibrinolysis much more potently than *trans*-AMCHA by blocking the active center of plasmin. It was also revealed that Tra–Lys–4-methoxycarbonylanilide (**14**) inhibited plasma kallikrein more potently than plasmin.[8] These results provided us with some ideas for designing specific inhibitors against various enzymes, and further extensive studies are now proceeding in our laboratory in order to obtain more potent and selective inhibitors not only against plasmin but also plasma kallikrein.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer (K-101AS, Kyowa Seimitsu). For thin-layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5, Rf^6 and Rf^7 values refer to the systems of $CHCl_3$, MeOH and AcOH (90:8:2), $CHCl_3$, MeOH and H_2O (89:10:1), $CHCl_3$, MeOH and H_2O (8:3:1, lower phase), *n*-BuOH, AcOH and H_2O (4:1:5, upper phase), *n*-BuOH, AcOH, pyridine and H_2O (4:1:1:2), *n*-BuOH, AcOH, pyridine and H_2O (1:1:1:1) and $CHCl_3$ and ether (4:1), respectively.

General Procedure for Synthesis of R–Lys(Z)–BZA [R: Z-NH(CH$_2$)$_4$CO–, Z-NH(CH$_2$)$_5$CO–, Z-NH–CH$_2$–⟨H⟩–CO– (*trans*), (*cis*), Z-NH–⟨H⟩~CO–, Z-NH–⟨H⟩~CO–] An acid chloride [prepared from the corresponding carboxylic acid derivative (2 mmol) and $SOCl_2$ (0.6 ml, 8 mmol) as usual] in $CHCl_3$ was added to an ice-cold solution of H–Lys(Z)–BZA·HCl [prepared from Boc–Lys(Z)–BZA[6] (0.70 g, 1.3 mmol) and 5.6 N HCl/dioxane (2.3 ml) as usual] in DMF (8 ml) containing Et_3N (0.36 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4, and concentrated to a small volume. The oily product in $CHCl_3$ (4 ml) was applied to a silica gel column (1.4 × 27 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (100 ml each) were collected, and the solvent of the effluent (tube Nos. 8—10) was removed by evaporation. Petroleum ether was added to the residue to give crystals, which were collected by filtration. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table V.

General Procedure for Synthesis of R–Lys–BZA [R: NH$_2$(CH$_2$)$_4$CO–, NH$_2$(CH$_2$)$_5$CO–, NH$_2$CH$_2$–⟨H⟩–CO– (*trans*), (*cis*), NH$_2$–⟨H⟩~CO–, NH$_2$–⟨H⟩~CO–] The corresponding N^{α}-Z-derivative (1.0 mmol) in EtOH (8 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford an amorphous powder. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table VI.

Boc–D-Lys(Z)–BZA A mixed anhydride [prepared from Boc–D-Lys(Z)–OH (5.7 g, 15 mmol) and ethyl chloroformate (1.4 ml, 15 mmol) as usual] in THF (200 ml) was added to an ice-cold solution of 4-benzoylaniline (2.6 g, 15 mmol) in THF (50 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (10 ml) was applied to a silica gel column (2.6 × 47.5 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (150 ml each) were collected. The solvent of the effluent (tube Nos. 8—13)

TABLE V. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of R–Lys(Z)–BZA

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
Z-NH-(CH$_2$)$_4$CO–	64.7	120—124	−27.0 (CHCl$_3$)	$C_{40}H_{44}N_4O_7$	69.3 (69.2	6.41 6.53	8.08 8.08)	0.64	
Z-NH-(CH$_2$)$_5$CO–	73.1	95—98	−28.3 (CHCl$_3$)	$C_{41}N_{46}N_4O_7$	69.7 (69.5	6.57 6.48	7.92 7.68)	0.72	
Z-NH-CH$_2$–⟨H⟩''''CO– (*trans*)	37.9	188—192	+2.7 (DMF)	$C_{43}H_{48}N_4O_7$	70.5 (70.2	6.55 6.62	7.65 7.78)	0.83	0.85
Z-NH-CH$_2$–⟨H⟩–CO– (*cis*)	50.9	78—85	−24.6 (MeOH)	$C_{43}H_{48}N_4O_7$	70.5 (70.5	6.55 6.57	7.65 7.57)	0.80	0.85
Z-NH–⟨H⟩~CO–	17.6	53—59	−36.6 (MeOH)	$C_{42}H_{46}N_4O_7$ 1.5H_2O	70.2 (70.3	6.45 6.83	7.79 7.72)	0.52	0.84
Z-NH–⟨H⟩~CO–	61.1	167—169	+2.9 (DMF)	$C_{42}H_{46}N_4O_7$	70.2 (70.4	6.45 6.45	7.79 7.81)	0.57	0.86

TABLE VI. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of R–Lys–BZA

Compound R		Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
						C	H	N	Rf^5	Rf^6
$NH_2(CH_2)_4CO-$	(1)	82.0	Amorphous	−30.3 (MeOH)	$C_{24}H_{32}N_4O_3$ ·2HCl	57.9 (57.6	6.90 6.98	11.3 11.0)	0.10	0.78
$NH_2(CH_2)_5CO-$	(2)	91.0	Amorphous	−35.1 (MeOH)	$C_{25}H_{34}N_4O_3$ ·2HCl	58.7 (58.5	7.11 7.21	11.0 10.8)	0.12	0.88
NH_2CH_2–⟨H⟩–CO– (*trans*)	(3)	91.3	89—92	−6.5 (MeOH)	$C_{27}H_{36}N_4O_3$ ·1.5H_2O	66.0 (66.1	7.99 7.98	11.4 11.1)	0.56	
NH_2CH_2–⟨H⟩–CO– (*cis*)	(4)	40.0	Amorphous	−44.0 (MeOH)	$C_{27}H_{36}N_4O_3$ ·1.5H_2O	66.0 (66.3	7.99 8.28	11.4 11.3)	0.54	
NH_2–⟨H⟩~CO–	(5)	51.9	Amorphous	−68.3 (MeOH)	$C_{26}H_{34}N_4O_3$ ·1.5H_2O	65.4 (65.6	7.75 7.44	11.7 11.5)	0.63	
NH_2–⟨H⟩~CO–	(6)	62.4	59—60	−25.7 (MeOH)	$C_{26}H_{34}N_4O_3$ ·H_2O	66.7 (66.5	7.69 7.68	12.0 11.7)	0.56	

was removed by evaporation. Petroleum ether was added to the residue to give crystals, yield 3.3 g (44.8%), mp 52—55 °C, $[\alpha]_D^{26}$ +9.8° (*c*=1.0, MeOH), Rf^1 0.65, Rf^3 0.92. *Anal.* Calcd for $C_{32}H_{37}N_3O_6$: C, 68.7; H, 6.66; N, 7.51. Found: C, 68.8; H, 6.71; N, 7.48.

Z–Tra–D-Lys(Z)–BZA An acid chloride [prepared from Z–Tra–OH (0.2 g, 0.86 mmol) and $SOCl_2$ (0.15 ml, 3.4 mmol) as usual] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–D-Lys(Z)–BZA·HCl [prepared from Boc–D-Lys(Z)–BZA (0.2 g, 0.39 mmol) and 5.6 N HCl/dioxane (0.7 ml) as usual] in DMF (54 ml) containing Et_3N (0.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvents, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 50 mg (17.5%), mp 133—135 °C, $[\alpha]_D^{26}$ −7.0° (*c*=0.1, DMF), Rf^2 0.38, Rf^3 0.61. *Anal.* Calcd for $C_{43}H_{48}N_4O_7$: C, 70.5; H, 6.55; N, 7.65. Found: C, 70.4; H, 6.58; N, 7.75.

H–Tra–D-Lys–BZA (7) Z–Tra–D-Lys(Z)–BZA (31.4 mg, 0.04 mmol) in MeOH (10 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford crystals, yield 10.0 mg (50.1%), mp 75—79 °C, $[\alpha]_D^{26}$ +8.5° (*c*=0.1, MeOH), Rf^5 0.45, Rf^6 0.79. *Anal.* Calcd for $C_{27}H_{36}N_4O_3$·1.5H_2O: C, 66.0; H, 7.91; N, 11.4. Found: C, 66.0; H, 7.64; N, 11.0.

Boc–Orn(Z)–BZA The title compound was synthesized from a mixed anhydride [prepared from Boc–Orn(Z)–OH (1.2 g, 3.3 mmol) and ethyl chloroformate (0.32 ml, 3.3 mmol) as usual] and 4-benzoylaniline (0.65 g, 3.3 mmol) by the same method as described in the synthesis of Boc–D-Lys(Z)–BZA. Yield 0.95 g (52.5%), mp 97—99 °C, $[\alpha]_D^{26}$ −13.4° (*c*=0.9, MeOH), Rf^1 0.69, Rf^2 0.80. *Anal.* Calcd for $C_{31}H_{35}N_3O_6$·0.5H_2O: C, 67.2; H, 6.50; N, 7.58. Found: C, 66.9; H, 6.44; N, 7.62.

Z–Tra–Orn(Z)–BZA The title compound was prepared from an acid chloride [prepared from Z–Tra–OH (0.1 g, 0.43 mmol) and $SOCl_2$ (0.08 ml, 1.7 mmol)] and H–Orn–BZA·HCl [prepared from Boc–Orn(Z)–BZA (0.1 g, 0.19 mmol) and 5.6 N HCl/dioxane (0.4 ml) as usual] by the same method as described in the synthesis of Z–Tra–D-Lys(Z)–BZA. Yield 0.10 g (65.3%), mp 152—160 °C, $[\alpha]_D^{26}$ −11.6° (*c*=0.3, DMF), Rf^1 0.63, Rf^2 0.56. *Anal.* Calcd for $C_{42}H_{46}N_4O_7$: C, 70.4; H, 7.38; N, 8.21. Found: C, 70.3; H, 7.74; N, 8.07.

H–Tra–Orn–BZA (8) Z–Tra–Orn(Z)–BZA (60.0 mg, 0.08 mmol) in DMF (12 ml) was hydrogenated over a Pd catalyst. Yield 29.9 mg (79.1%), mp 99—104 °C, $[\alpha]_D^{26}$ −11.3° (*c*=0.5, DMSO), Rf^5 0.47. *Anal.* Calcd for $C_{26}H_{34}N_4O_3$·2.5H_2O: C, 63.1; H, 7.88; N, 11.0. Found: C, 63.1; H, 7.90; N, 11.3.

General Procedure for Synthesis of Boc–Lys(Z)–R [R: –N⟨⟩–COOC$_2$H$_5$, –N(tetrahydroquinolyl), –NH–(tetrahydronaphthyl), –NH–⟨⟩–COOCH$_3$] A mixed anhydride [prepared from Boc–Lys(Z)–OH (3.8 g, 10 mmol) and ethyl chloroformate (0.96 ml, 10 mmol) as usual] in THF (60 ml) was added to an ice-cold solution of the corresponding amino component (0.01 mol) in THF (30 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (10 ml) was applied to a silica gel column (2.6 × 47.5 cm), equilibrated and eluted with $CHCl_3$. Individual fractions (150 ml each) were collected. The solvent of the effluent (tube Nos. 5—10) was removed by evaporation. Petroleum ether was added to the residue to give crystals. Yield, mp, $[\alpha]_D^{26}$ value, *Rf* values and elemental analysis are summarized in Table VII.

General Procedure for Synthesis of Z–Tra–Lys(Z)–R [R: –N⟨⟩–COOC$_2$H$_5$, –N⟨⟩–CH$_2$–⟨⟩, –N(tetrahydroquinolyl), –NH–⟨⟩–NO$_2$, –NH–(tetrahydronaphthyl), –NH–⟨⟩–COOCH$_3$, –NH–⟨⟩–COCH$_3$] An acid chloride [prepared from Z–Tra–OH (1.5 g, 6.8 mmol) and $SOCl_2$ (1.2 ml, 27.2 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–R·HCl [prepared from the corresponding Boc–Lys(Z)–R (2.3 mmol) and 5.5 N HCl/dioxane (4.2 ml) as usual] in DMF (10 ml) containing Et_3N (0.6 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and H_2O were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table VIII.

General Procedure for Synthesis of Tra–Lys–R [R: –N⟨⟩–COOC$_2$H$_5$, –N⟨⟩–CH$_2$–⟨⟩, –N(tetrahydroquinolyl), –NH–⟨⟩–NO$_2$, –NH–(tetrahydronaphthyl), –NH–⟨⟩–COOCH$_3$, –NH–⟨⟩–COCH$_3$] The corresponding protected compound (34.8 mg, 0.05 mmol) in DMF (6 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford an amorphous powder. In the case of the *p*NA derivative, a Z group was removed by HBr/AcOH. Yield, mp, $[\alpha]_D$ value, *Rf* values and elemental analysis are summarized in Table IX.

Z–Tra–Lys(Z)–4-Benzoylphenyl Ester An acid chloride [prepared from Z–Tra–OH (1.2 g, 5.5 mmol) and $SOCl_2$ (0.9 ml, 22 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–4-benzoylphenyl ester·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.3 g, 0.54 mmol) and 5.6 N HCl/dioxane (1 ml)] in DMF (5 ml) containing Et_3N (0.15 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and reprecipitated from AcOEt and ether, yield 0.16 g (40.4%), mp 109—113 °C, $[\alpha]_D^{26}$

TABLE VII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Boc–Lys(Z)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
–N, –COOC$_2$H$_5$	43.2	Oil	−6.6 (MeOH)	$C_{27}H_{41}N_3O_7 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.54	0.67
–NH	27.8	Oil	−4.1 (MeOH)					0.64	0.76
–NH–	64.8	95—96	−13.4 (MeOH)	$C_{29}H_{39}N_3O_5$	68.3 (68.3	7.71 7.84	8.25 8.22)	0.69	0.80
–NH– –COOCH$_3$	39.0	118—119	−25.7 (CHCl$_3$)	$C_{27}H_{35}N_3O_7 \cdot 1/2H_2O$	62.1 (62.4	6.89 6.89	8.04 8.03)	0.60	0.65

TABLE VIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Tra–Lys(Z)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
–N, –COOC$_2$H$_5$	28.3	Oil	+28.3 (MeOH)	$C_{38}H_{52}N_4O_8$	65.9 (65.5	7.57 7.38	8.09 8.03)	0.52	0.50
–N –CH$_2$–	47.3	Oil	+2.7 (MeOH)	$C_{42}H_{54}N_4O_6$	71.0 (70.9	7.66 7.66	7.88 7.92)	0.60	0.64
–N	28.3	94—96	+42.9 (DMF)	$C_{39}H_{48}N_4O_6$	70.0 (70.1	7.23 7.18	8.38 8.13)	0.45	0.94
–NH– –NO$_2$	61.3	199—205	+5.9 (MeOH)	$C_{36}H_{43}N_5O_8$	64.2 (64.3	6.43 6.50	10.4 10.4)	0.81	0.86
–NH–	60.5	210—213	−6.0 (DMF)	$C_{40}N_{50}N_4O_6$	70.4 (70.3	7.38 7.48	8.21 8.07)	0.74	0.80
–NH– –COOCH$_3$	68.4	203—204	−33.4 (CHCl$_3$)	$C_{38}H_{46}N_4O_8$	66.5 (66.2	6.70 6.91	8.16 8.06)	0.70	
–NH– –COCH$_3$	71.2	202—204	−4.0 (DMF)	$C_{38}H_{46}N_4O_7$	68.1 (67.8	6.86 6.86	8.35 8.19)	0.63	0.78

−20.3° (c=0.4, CHCl$_3$), Rf^1 0.65, Rf^2 0.83. *Anal.* Calcd for $C_{43}H_{47}N_3O_8$: C, 70.4; H, 6.47; N, 5.73. Found: C, 70.2; H, 6.41; N, 5.74.

H-Tra-Lys-4-Benzoylphenyl Ester (16) Z–Tra–Lys(Z)–4-Benzoylphenyl ester (0.077 g, 0.1 mmol) was dissolved in 25% HBr/AcOH (0.2 ml, 0.6 mmol). The reaction mixture was stored at room temperature for 30 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. The resulting HBr salt in EtOH (4 ml) containing Et$_3$N (0.28 ml, 0.2 mmol) was kept at room temperature for 10 min and purified by column chromatography. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 45—56), ether was added to the residue to give crystals, yield 0.026 g (55.9%), mp 75—78 °C, $[\alpha]_D^{26}$ −11.5° (c=0.1, MeOH), Rf^5 0.42, Rf^6 0.88. *Anal.* Calcd for $C_{27}H_{35}N_3O_4 \cdot 2H_2O$: C, 67.1; H, 8.15; N, 8.69. Found: C, 66.9; H, 8.38; N, 8.54.

Z-Tra-Lys(Z)-4-Benzoylanilinomethyl Ketone 4-Benzoylaniline (0.23 g, 1.2 mmol), NaHCO$_3$ (0.1 g, 1.2 mmol), NaI (0.18 g, 1.2 mmol) and Boc–Lys(Z)–CH$_2$Cl (0.48 g, 1.2 mmol) were dissolved in DMF (15 ml). The reaction mixture was stirred at 45 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% NaHCO$_3$ and H$_2$O, dried over Na$_2$SO$_4$ and evaporated down. Petroleum ether was added to the residue to give Boc–Lys(Z)–4-benzoylanilinomethyl ketone as an amorphous powder [yield 0.24 g (35.3%), $[\alpha]_D^{26}$ −21.2° (c=1.2, CHCl$_3$)]. An acid chloride [prepared from Z–Tra–OH (0.39 g, 1.7 mmol) and SOCl$_2$ (1.2 ml, 7 mmol)] in CHCl$_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–4-benzoylanilinomethyl ketone·HCl [prepared from the corresponding N^{α}-Boc-derivative (0.25 g, 0.44 mmol) obtained above and 5.6 N HCl/dioxane (0.81 ml)] in DMF (8 ml) containing Et$_3$N (0.12 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na$_2$CO$_3$ and H$_2$O, dried over Na$_2$SO$_4$ and evaporated down. The oily product in CHCl$_3$ (3 ml) was applied to a silica gel column (2.4 × 32 cm), equilibrated and eluted with CHCl$_3$. Individual fractions (50 ml each) were collected. The solvent of the effluent (tube Nos. 2—7) was removed by evaporation. Petroleum ether was added to the residue to afford an amorphous powder, yield 0.1 g (31.1%), $[\alpha]_D^{26}$ −2.5° (c=0.9, CHCl$_3$), Rf^1 0.50, Rf^2 0.76. *Anal.* Calcd for $C_{44}H_{50}N_4O_9 \cdot H_2O$: C, 69.1; H, 6.80; N, 7.33. Found: C, 69.3; H, 6.60; N, 7.06.

H-Tra-Lys-4-Benzoylanilinomethyl Ketone (17) Z–Tra–Lys(Z)–4-ben-

TABLE IX. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of H–Tra–Lys–R

Compound R		Yield (%)	mp (°C)	$[\alpha]_D$ (°) (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
						C	H	N	Rf^5	Rf^6
–N ... $COOC_2H_5$	(9)	59.2	Amorphous	−23.3 (MeOH)	$C_{22}H_{40}N_4O_4 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.56	
–N ... CH_2–	(10)	51.4	Amorphous	−8.0 (MeOH)	$C_{26}H_{42}N_4O_2 \cdot 3.5H_2O$	61.8 (61.8	9.76 9.53	11.1 10.9)	0.55	
–N	(11)	59.2	Amorphous	−9.8 (MeOH)	$C_{23}H_{36}N_4O_2 \cdot H_2O$	67.4 (67.5	7.33 7.18	8.05 8.03)	0.56	
–NH– ... NO_2	(12)	61.3	Amorphous	−10.4 (MeOH)	$C_{20}H_{31}N_5O_4 \cdot 2HBr \cdot 4H_2O$	38.2 (37.9	6.52 6.33	11.1 10.9)	0.62	
–NH–	(13)	59.2	Amorphous	−8.8 (MeOH)	$C_{24}H_{38}N_4O_2 \cdot H_2O$	66.7 (66.6	9.25 9.52	13.0 12.9)	0.56	
–NH– ... $COOCH_3$	(14)	56.6	48—50	−9.3 $(CHCl_3)$	$C_{22}H_{34}N_4O_4 \cdot 3H_2O$	56.0 (56.3	7.47 7.69	10.9 10.6)	0.39	0.71
–NH– ... $COCH_3$	(15)	66.3	164 (dec.)	−10.6 (DMF)	$C_{22}H_{34}N_4O_3 \cdot H_2O$	62.9 (63.1	8.57 8.35	13.3 13.0)	0.39	

zoylanilinomethyl ketone (0.046 g, 0.061 mmol) was dissolved in 25% HBr/AcOH (0.1 ml, 0.37 mmol) and the reaction mixture was stored at room temperature for 30 min. Ether was added to the solution to afford a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the corresponding hydrobromide in EtOH (1 ml) containing Et_3N (0.17 ml, 0.12 mmol) was allowed to stand at room temperature for 10 min and applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 47—58), ether was added to the residue to give an amorphous powder, yield 0.11 g (37%), $[\alpha]_D^{26}$ −0.38° (*c*=0.5, MeOH), Rf^5 0.47, Rf^3 0.70. *Anal.* Calcd for $C_{28}H_{38}N_4O_3 \cdot HBr \cdot 3H_2O$: C, 54.9; H, 7.34; N, 9.13. Found: C, 54.6; H, 7.69; N, 9.44.

Z–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone 4-Benzoylphenol (0.26 g, 1.3 mmol), $NaHCO_3$ (0.11 g, 1.3 mmol), NaI (0.039 g, 1.3 mmol) and Boc–Lys(Z)–CH_2Cl (0.53 g, 1.3 mmol) were dissolved in DMF (10 ml), and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give the title compound as an amorphous powder [yield 0.59 g (80.4%), $[\alpha]_D^{26}$ +4.47° (*c*=0.8, $CHCl_3$)]. An acid chloride [prepared from Z–Tra–OH (0.9 g, 4.1 mmol) and $SOCl_2$ (1.9 ml, 16 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^α-Boc-derivative (0.7 g, 1.2 mmol) and 5.6 N HCl/dioxane (2 ml)] in DMF (5 ml) containing Et_3N (0.34 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 0.17 g (19.0%), mp 147—149 °C, $[\alpha]_D^{26}$ −38.0° (*c*=1.0, DMSO), Rf^1 0.80, Rf^2 0.85. *Anal.* Calcd for $C_{44}H_{49}N_3O_8$: C, 70.7; H, 6.56; N, 5.62. Found: C, 70.4; H, 6.75; N, 5.79.

H–Tra–Lys–4-Benzoylphenoxymethyl Ketone (18) Z–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (0.08 g, 0.1 mmol) was dissolved in 25% HBr/AcOH (0.2 ml, 0.6 mmol) and the solution was kept at room temperature for 30 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the resulting hydrobromide in EtOH (2 ml) containing Et_3N (0.028 ml, 0.2 mmol) was applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 44—60), ether was added to the residue to give an amorphous powder, yield 0.045 g (93.9%), $[\alpha]_D^{26}$ −15.6° (*c*=0.8, MeOH), Rf^5 0.37, Rf^6 0.68. *Anal.* Calcd for $C_{28}H_{37}N_3O_4 \cdot HBr \cdot 3H_2O$: C, 54.8; H, 7.16; N, 6.84. Found: C, 54.3; H, 7.25; N, 6.67.

Boc–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone The title compound was prepared by the essentially same method as described in the synthesis of Z–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone from a mixed anhydride [prepared from Boc–Tra–OH (0.15 g, 0.58 mmol) and ethyl chloroformate (0.056 ml, 0.58 mmol)] and H–Lys(Z)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^α-Boc-derivative (0.2 g, 0.42 mmol) and 5.6 N HCl/dioxane (0.74 ml)]. Yield 0.067 g (22.4%), mp 99—102 °C, $[\alpha]_D^{26}$ −6.5° (*c*=0.2, $CHCl_3$), Rf^1 0.63, Rf^2 0.97. *Anal.* Calcd for $C_{41}H_{51}N_3O_8 \cdot 0.5H_2O$: C, 68.2; H, 7.20; N, 5.81. Found: C, 68.1; H, 6.95; N, 5.87.

H–Tra–Lys(Z)–4-Benzoylphenoxymethyl Ketone (19) Boc–Tra–Lys(Z)–4-benzoylphenoxymethyl ketone (0.052 g, 0.075 mmol) was dissolved in 5.6 N HCl/dioxane (0.1 ml, 0.38 mmol). After 1 h at room temperature, ether was added to the solution to afford a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*. A solution of the resulting hydrochloride in EtOH (1 ml) containing a saturated Na_2CO_3 solution (1 ml) was applied to a column of Sephadex LH-20 (3.2 × 53 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. After removal of the solvent of the effluent (tube Nos. 19—26), ether was added to the residue to give crystals, yield 0.03 g (66.5%), mp 109—114 °C, $[\alpha]_D^{26}$ −2.7° (*c*=0.2, MeOH), Rf^4 0.42, Rf^5 0.70. *Anal.* Calcd for $C_{36}H_{43}N_3O_6 \cdot 2.5H_2O$: C, 65.7; H, 7.29; N, 6.38. Found: C, 65.4; H, 7.58; N, 6.29.

Z–Tra–Lys(Boc)–4-Benzoylphenoxymethyl Ketone 4-Benzoylphenol (0.73 g, 3.7 mmol), $NaHCO_3$ (0.31 g, 3.7 mmol), NaI (0.55 g, 3.7 mmol) and Z–Lys(Boc)–CH_2Cl (1.5 g, 3.7 mmol) were dissolved in DMF (15 ml) and the reaction mixture was stirred at 45 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give Z–Lys(Boc)–4-benzoylphenoxymethyl ketone as an oily product (yield 70.6%), $[\alpha]_D^{26}$ −11.5° (*c*=1.1, $CHCl_3$)]. An acid chloride [prepared from Z–Tra–OH (0.17 g, 0.57 mmol) and $SOCl_2$ (0.27 ml, 2.3 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Boc)–4-benzoylphenoxymethyl ketone·HCl [prepared from the corresponding N^α-Z-derivative (0.22 g, 0.38 mmol) in MeOH (10 ml) containing 1 N HCl (0.38 ml) by catalytic hydrogenation] in DMF (10 ml) containing Et_3N (0.11 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract

was washed with 10% citric acid, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 0.064 g (23.4%), mp 85—87 °C, $[\alpha]_D^{26}$ +0.8° (c=0.5, $CHCl_3$), Rf^1 0.71, Rf^2 0.72. *Anal.* Calcd for $C_{41}H_{51}N_3O_8$: C, 69.0; H, 7.15; N, 5.89. Found: C, 68.8; H, 7.35; N, 6.00.

Z–Tra–Lys–4-Benzoylphenoxymethyl Ketone (20) The title compound was prepared by the essentially same method as described in the synthesis of **19** from Z–Tra–Lys(Boc)–4-benzoylphenoxymethyl ketone (0.054 g, 0.075 mmol) and 5.6 N HCl/dioxane (0.1 ml). Yield 0.021 g (44.6%), mp 75—77 °C, $[\alpha]_D^{26}$ −10.0° (c=0.5 MeOH), Rf^4 0.31, Rf^5 0.61. *Anal.* Calcd for $C_{36}H_{43}N_3O_6 \cdot 3H_2O$: C, 64.8; H, 7.34; N, 6.29. Found: C, 65.1; H, 7.52; N, 6.39.

Assay Procedure IC_{50} values were determined according to the procedure described in the previous report.[6]

References and Notes

1) The customary L indication for amino acids is omitted; only D isomers are indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2584 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Tra, *trans*-4-aminomethylcyclohexanecarbonyl; Eac, ε-aminocaproyl; *p*NA, *p*-nitroanilide; BZA, 4-benzoylanilide; ACA, 4-acetylanilide; BPP, 4-benzylpiperidine amide; AcOH, acetic acid; DMF, *N,N*-dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; S-2251, H–D-Val–Leu–Lys–*p*NA; Fn, fibrin; Fg, fibrinogen; Tos, tosyl; Orn, ornithine.
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Identification of Plant Factors Inducing Virulence Gene Expression in *Agrobacterium tumefaciens*

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In the natural gene-transfer system of *Agrobacterium tumefaciens*, the expression of virulence (*vir*) gene of Ti (tumor inducing) plasmid is essential for the subsequent transferred deoxyribonucleic acid (T-DNA) transfer from bacterium to plant cells. *vir* gene expression is induced by signal compounds from plants such as acetosyringone and α-hydroxyacetosyringone which were isolated from hairy root cultures of *Nicotiana tabacum*. In a search for intrinsic signal compounds in plants, acetosyringone and α-hydroxyacetosyringone were identified as the *vir*-inducing compounds of the hairy root cultures of *Atropa belladonna*. The signal compounds of seedlings of *Pisum sativum* were identified as sinapyl and coniferyl alcohols. The quantities of these *vir*-inducing compounds were significantly increased when plant tissues were wounded, though the degree of increase was markedly different in each plant.

Keywords *Agrobacterium tumefaciens*; α-hydroxyacetosyringone; acetosyringone; sinapyl alcohol; coniferyl alcohol; *vir* gene; *Atropa belladonna*; *Pisum sativum*; induction; signal compound

Introduction

Agrobacterium tumefaciens is a phytopathogenic bacterium harboring Ti (tumor inducing) plasmid which is responsible for the induction of crown galls, a tumorous tissue, in plants. When a plant is infected with *A. tumefaciens*, the transferred deoxyribonucleic acid (T-DNA) region of Ti plasmid is transferred and integrated into plant genomic DNA to induce crown galls.[1] This natural gene-transfer system has been extensively used as a tool to transfer foreign genes into plants. The use of Ti plasmids for gene-transfer experiments is limited to dicotyledon plants, since, in general, monocotyledon plants are not susceptible to *Agrobacterium* infection. The mechanism of T-DNA transfer has not been fully clarified, but a gene cluster at virulence (*vir*) loci (*A*, *B*, *C*, *D*, *E*, and *G*) which are located outside of T-DNA plays a critical role in the gene transfer, and their translation products take part in the following T-DNA transfer into plant cells.[2,3] VirA protein is constitutionally expressed and acts as a receptor of signal compounds which is essential in the signal transduction process. Co-cultivation experiments of plant cells with *A. tumefaciens* demonstrated that the expression of *vir* genes (*virB*, *-C*, *-D*, *-E*, and *-G*) of *A. tumefaciens* was induced at a high level in the presence of plant cells, indicating that signal compounds from the plant activated *vir* gene expression.[4] *vir* gene activating compounds of *Nicotiana tabacum* were identified as the derivatives of acetophenones, acetosyringone (AS) and α-hydroxyacetosyringone (HOAS).[5]Although a considerable number of compounds which have similar functional groups to AS were reported to possess *vir*-inducing activity, AS and HOAS were the only intrinsic *vir*-inducing compounds of plants up to quite recently.[5-7] Spencer *et al.* reported recently the isolation of methyl syringate as an intrinsic *vir*-inducing factor in grapevine.[8] Our interest in this phenomenon is to clarify the intrinsic signal compounds in the plants which are infected by *A. tumefaciens* to form crown galls.

In order to identify the intrinsic signal compounds of plants other than *N. tabacum*, a variety of materials such as the extracts of medicinal plants,[9] cultured plant cells with or without elicitor induction[10] and hairy root cultures[11] were tested for their *vir* gene inducing activity. A *lacZ*::*virB* fused strain of *A. tumefaciens* was used to monitor *vir*-inducing activity, which was quantified with β-galactosidase activity by measuring exprssion of the β-galactosidase gene inserted in the *virB* region. Among the samples tested the medium of hairy root cultures of *Atropa belladonna* was found to induce *vir* gene expression most significantly. The *vir*-inducing activity was also detected in the aqueous extracts of seedlings of *Pisum sativum*. In a previous communication, we reported briefly the isolation and identification of HOAS and AS as the plant *vir*-inducing factors of hairy root cultures of *A. belladonna*.[12] It has also been shown that wounding of plant tissues caused a significant increase in the level of signal compounds. This paper reports full details of these studies as well as the identification of *vir* gene activating factors in seedlings of *P. sativum*.

Results

vir-Inducing Compounds of Hairy Roots of *A. belladonna* and Seedlings of *P. sativum* A medium of hairy root cultures of *A. belladonna* was concentrated and fractionated with a XAD-2 column by eluting with water and methanol. *vir* gene inducing activity was monitored in each fraction. The activity was observed in the methanolic fraction of the XAD-2 column, which was further chromatographed on a reverse phase Lobar column to give two active fractions. The *vir*-inducing activity recovered in methanol-eluted fractions was much lower than the original activity in the culture medium. The activity of the rather non-polar fractions was markedly enhanced by the addition of water-eluted fractions in the XAD-2 column chromatography, though the latter fractions themselves had no *vir*-inducing activity. The potentiating factor having a synergistic effect was identified as inositol which is one component of Murashige–Skoog's medium. Following this finding, various kinds of sugar samples were tested for their potentiating effect on the *vir*-inducing activity of AS and the results were reported briefly in our previous communication.[12] This phenomenon will be discussed in details in a forthcoming paper.

Two *vir*-inducing compounds which were isolated after reverse phase high performance liquid chromatography (HPLC) were tentatively designated as BL-1 and BL-2, these were thought to be AS and HOAS, since *A. belladonna* is a solanaceous plant. BL-1, a major compound, was finally identified as HOAS and BL-2, a minor one, as AS by the

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comparison of ultraviolet (UV), mass (MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectra with those of standard samples. A standard sample of HOAS was not available, so HOAS was synthesized chemically by the hydrolysis of diacetyl HOAS which had been prepared from AS by a known method.[13] It may be worth noting here that the MS spectra of HOAS and methyl syringate are very similar and their identification requires other methods than MS, such as NMR or HPLC.

In seedlings of *P. sativum*, *vir*-inducing activity was detected in the aqueous extracts. The extracts were applied on a XAD-2 column and the active compounds were eluted with methanol. Further separation of the active fractions with a reverse phase Lobar column and HPLC afforded two active compounds designated as PS-1 and PS-2. PS-1 was finally identified as sinapyl alcohol and PS-2 as coniferyl alcohol by investigation of the UV, MS and ^{1}H-NMR spectra. Since a sample of sinapyl alcohol was not available due to its instability, a standard sample was synthesized from ethyl acetylsinapate by lithium aluminum hydride reduction according to a known method.[14]

Figure 2 shows a comparison of *vir*-inducing activity of HOAS, AS, sinapyl alcohol and coniferyl alcohol. The activities of the four compounds follow similar patterns in a concentration-dependent manner. From 5 to 100 μM it was seen that the *vir*-inducing activity increased outstandingly. Although AS has the highest activity among them, sinapyl alcohol also exhibited a higher activity.

acetosyringone (AS) α-hydroxyacetosyringone (HOAS)

sinapyl alcohol coniferyl alcohol

Fig. 1. The Structures of *vir*-Inducing Compounds Isolated from Hairy Root Cultures of *A. belladonna* and Seedlings of *P. sativum*

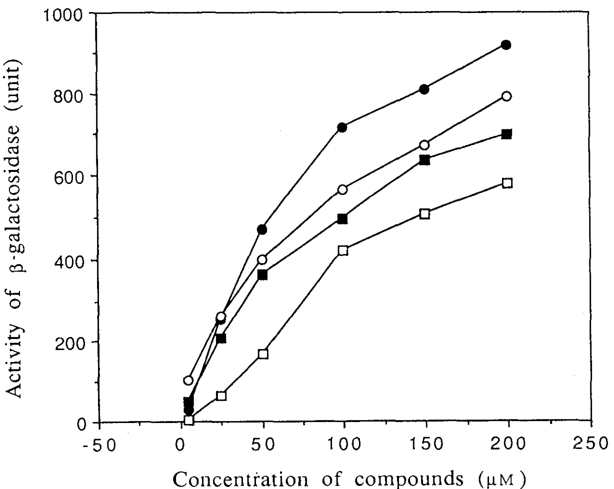

Fig. 2. A Comparison of *vir*-Inducing Activity of α-Hydroxyacetosyringone (HOAS), Acetosyringone (AS), Sinapyl Alcohol (Sina. alc.) and Coniferyl Alcohol (Coni. alc.)

The standard compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in induction buffer[15] to a final concentration of 0.4% DMSO. Induction assay was done as described in Experimental. Each point on the activity curve represents the average of the results of two to five independent experiments. —●—, AS; —□—, HOAS; —○—, Sina. alc.; —■—, Coni. alc.

Effect of Wounding on the Contents of Signal Compounds The transformation of plants by *Agrobacterium* is usually carried out with wounded plants. Stachel *et al.* demonstrated that an increase of AS and HOAS occurred after *N. tabacum* plants were wounded.[5] The observed facts support the general view that *Agrobacterium* infection needs some stimuli from wounded cells. To obtain further support for this view, we measured the time course changes of the *vir*-inducing compounds after wounding. The response of two plant systems used in the time course studies are quite different as shown in Tables I and II. Production of the *vir*-inducing compounds was stimulated by wounding of plant cells. The effect of wounding lasted rather longer in hairy roots of *A. belladonna* but was a transient phenomenon in seedlings of *P. sativum*. It is worthy of note here that the contents of HOAS and AS in hairy root cultures of *A. belladonna* were higher in the culture media than those in hairy roots in all the experiments. As suggested by Stachel *et al.* this may reflect the nature of the

Table I. Wounded Plant Tissue Effects on the Production of HOAS and AS in Hairy Root Cultures of *A. belladonna*

Treatment	α-Hydroxyacetosyringone (HOAS)		Acetosyringone (AS)	
	Medium	Hairy roots	Medium	Hairy roots
Not wounded	72.2	27.9	13.0	7.0
Wounded 24 h	80.5	28.5	20.4	13.6
Wounded 48 h	302.0	44.0	11.0	9.2
Wounded 72 h	551.0	69.4	12.7	10.4

Samples were prepared as described in Experimental. They were analyzed on ODS-80Tm column (4.6 mm i.d. × 15 cm) eluted with 20% CH_3OH 0.1% CH_3COOH (for HOAS) or 30% CH_3OH 0.1% CH_3COOH (for AS) at a flow rate of 0.8 ml/min; TOYO SODA CCPM with TOYO SODA UV-8000 was used as a monitor at 280 nm. The contents were calculated by a standard curve. Each value represents the average of the results of three independent samples. The values were shown as μg/g of dry hairy roots.

Table II. Wounded Plant Tissue Effects on the Production of Sinapyl Alcohol and Coniferyl Alcohol in Hypocotyls of *P. sativum*

Treatment	Sinapyl alcohol	Coniferyl alcohol
Not wounded	5.85	7.83
Wounded 24 h	6.55	12.80
Wounded 48 h	16.92	13.57
Wounded 72 h	5.15	8.60

Samples were prepared as described in Experimental. They were analyzed on ODS-80Tm column (4.6 mm i.d. × 15 cm) eluted with 26% CH_3OH 0.1% CH_3COOH at a flow rate of 0.8 ml/min; TOYO SODA CCPM with TOYO SODA UV-8000 was used as a monitor at 280 nm. The contents were calculated by a standard curve. Each value represents the average of the results of three independent samples. The values were shown as μg/g of dry weight.

vir-inducing compounds as being exudate-specific,[5] but not leakage-specific. The content of HOAS in the hairy root cultures of *A. belladonna* is much higher than that of AS. This is unlike *N. tabacum* where HOAS and AS were present at nearly the same concentration.[5] Higher *vir*-inducing activity of AS and HOAS and long-lasting production after wounding may be the reason for the high susceptibility of *N. tabacum* and *A. belladonna* for *Agrobacterium* infection. In contrast to HOAS and AS of hairy roots of *A. belladonna*, in the hypocotyls of *P. sativum* the contents of sinapyl and coniferyl alcohols are much lower, at a level equivalent to that of AS in *A. belladonna*. However, the *vir*-inducing activity of sinapyl alcohol is comparable to AS and that of coniferyl alcohol is considerably higher than that of HOAS.

Discussion

Before Stachel *et al.* isolated AS and HOAS from hairy roots of *N. tabacum*, these acetophenones had not been reported as plant constituents.[5] They seem to have rather narrow distribution in the plant kingdom. Contrary to this, it is taken for granted that C_6–C_3 (sinapyl and coniferyl) alcohols are building units of lignin and distributed much widely among plants. Of the five intrinsic signal compounds which have so far been isolated from plants, C_6–C_3 alcohols, sinapyl and coniferyl alcohols would be the most general intrinsic signal compounds in plants. Their secretory nature is not clear due to the experimental condition with seedlings of *P. sativum*. The C_6–C_3 alcohols are significantly different from AS and HOAS in this aspect, since they were not detected in the medium of hairy root cultures of *A. belladonna* after wounding. It is reasonably assumed that the C_6–C_3 alcohols are produced for the synthesis of lignin as a response to wounding and also act as signal compounds for *Agrobacterium* infection. The latter function was probably acquired by *Agrobacterium* during the course of co-evolution of plants and bacteria. HOAS and AS are also present in normal root cultures of *A. belladonna* and secreted into the culture medium (data are not shown), indicating that they are normal plant constituents as are the C_6–C_3 alcohols, and their production and exudation is induced by wounding of the plants. The reason the production of HOAS and AS is induced by wounding and what their function is in plants in normal and wounded tissues of *A. belladonna* are still unknown. The acetophenones may take part in certain defense or repair mechanisms of the plants as the C_6–C_3 alcohols do in plants. This plant and bacteria relationship is quite well organized, since *Agrobacterium* infection occurs only in wounded or traumatized tissues.

Experimental

Melting points were determined on a Yanagimoto MP-S3 micromelting point apparatus and are uncorrected. UV spectra were recorded with a Hitachi model 100-60 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with JEOL JNM-GSX 400 and 500 spectrometers. MS spectra were recorded on a JEOL JMS-D 300 mass spectrometer. Column chromatography was carried out on Kieselgel 60 (MERCK; 230—400 mesh) and MCI-gel CHP-20 (XAD-2, 37—75 μm). Reverse phase chromatography was carried out on an octadecyl silica (ODS) Lobar column (Lichroprep RP-18 MERCK 310-25). Preparative and analytical HPLC were carried out on a TOYO SODA CCPM HPLC system (detector, TOYO SODA UV-8000 ultraviolet monitor) using TSK gel ODS-80Tm column.

Hairy Root Cultures of *A. belladonna* Hairy root cultures of *A. belladonna* transformed with a strain of *Agrobacterium rhizogenes* ATCC 15834 were grown in liquid Murashige and Skoog (MS) Medium. Hairy roots were grown in 150 ml of medium in 500 ml flasks at 26 °C at 90 rmp and subcultured each 4 weeks.

Seedlings of *P. sativum* Seeds of *P. sativum* were soaked in water for 12 h and sowed on cotton infiltrated with water. The seeds were left to germinate in the dark at room temperature.

***vir* Gene Expression Assay** Bacterial strain A384 pSM243 (locus *virB*) was supplied by Prof. E. W. Nester of University of Washington. Bacterial cultures were grown in LBMG liquid medium[16] supplemented with kanamycin and ampicillin at a concentration of 50 μg/ml. After 48 h culture they were centrifuged and resuspended in induction buffer for an absorbance of 1.0 optical density (OD) units per ml at 600 nm. The bacterial suspension (0.2 ml) was added to the solution of 1.8 ml induction buffer containing test sample and the culture was incubated at 28—30 °C for 17 h. Half of the harvested bacteria were suspended in 1 ml of Z. Buffer[17] and bacterial density was measured at 600 nm (1 cm). The cell suspension (1 ml) was vigorously mixed with 0.3 ml of $CHCl_3$ and 0.2 ml of sodium dodecyl sulfate (SDS) at 0.05%. The reaction was started by adding 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside at 4 mg/ml. After incubation at 37 °C for 10 min, the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. After centrifugation, the absorbance at 420 nm (1 cm) of the supernatant was measured. Specific units of β-galactosidase activity were calculated using the formula $A_{420} \times 1000/A_{600}$ and are described as units per bacterial cell.

Isolation of HOAS and AS from Hairy Root Cultures of *A. belladonna* The culture medium of hairy roots was removed on 20 d after subculturing to obtain 2500 ml of medium. The culture medium was concentrated to 210 ml at a temperature below 50 °C, then passed through a column of XAD-2 (bed volume 1.6 × 11 cm). The column was step-wisely eluted with 50 ml of distilled water and 100 ml of CH_3OH. CH_3OH-eluted fraction was evaporated. Distilled water (25 ml) was added and extracted three times with 80% volume of benzene. Benzene layer was evaporated to dryness and the residue was dissolved in 40% CH_3OH. It was chromatographed on a reverse phase Lobar column using 35% CH_3OH and two active fractions were obtained. Each of the active fractions was respectively chromatographed on HPLC with a ODS-80Tm column (7.8 mm i.d. × 30 cm) using 30% CH_3OH and 40% CH_3OH to give two *vir*-inducing compounds, HOAS and AS.

HOAS A white amorphous solid. UV $\lambda_{max}^{CH_3OH}$ nm: 298. MS *m/z*: 212 (M^+), 181, 153. ^{1}H-NMR ($CDCl_3$) δ: 3.97 (s, 6H), 4.83 (s, 2H), 6.05 (s, 1H), 7.19 (s, 2H). Spectra of the isolated compound were the same as those of the synthetic compound.

Synthesis of HOAS The HOAS was synthesized from diacetate of HOAS[13] by hydrolysis with 5% HCl in 70% EtOH at 80 °C for 1.5 h. The product was isolated by column chromatography on silica gel and crystallized from benzene. A white needle. mp 109—110 °C *Anal.* Calcd for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70. Found: C, 56.25; H, 5.66. ^{1}H-NMR ($CDCl_3$) δ: 3.97 (s, 6H), 4.83 (s, 2H), 6.05 (s, 1H), 7.20 (s, 2H). ^{13}C-NMR ($CDCl_3$): 56.6 (2C), 64.8 (1C), 105.1 (2C), 124.9 (1C), 140.7 (1C), 147.1 (2C), 196.6 (1C). MS *m/z*: 212 (M^+), 181, 153.

AS An amorphous solid. UV $\lambda_{max}^{CH_3OH}$ nm: 298. MS *m/z*: 196 (M^+), 181, 153. ^{1}H-NMR ($CDCl_3$) δ: 2.58 (s, 3H), 3.96 (s, 6H), 7.25 (s, 2H). The spectra were the same as those of a standard compound.

Isolation of Sinapyl Alcohol and Coniferyl Alcohol from Seedlings of *P. sativum* Seedlings were harvested after the seeds had been sowed on moist cotton for 10 d. Fresh seedlings (56 g) were extracted with water at 120 °C by autoclaving. The extract was applied on a XAD-2 column, washed with water and then eluted with CH_3OH. CH_3OH fraction was concentrated under reduced pressure and dissolved in 40% CH_3OH. It was chromatographed on a reverse phase Lobar column by eluting with 40% CH_3OH to give two active fractions. Each active fraction was chromatographed again on HPLC (ODS-80Tm column 7.8 mm i.d. × 30 cm) and eluted with 40% CH_3OH to obtain two *vir*-inducing compounds, sinapyl alcohol and coniferyl alcohol.

Sinapyl Alcohol A white amorphous solid. UV $\lambda_{max}^{CH_3OH}$ nm: 272. MS *m/z*: 210 (M^+), 182, 167, 149. ^{1}H-NMR ($CDCl_3$) δ: 3.91 (s, 6H), 4.31 (d, 2H, J=6 Hz), 5.53 (s, 1H), 6.24 (dt, 1H, J=16, 6 Hz), 6.53 (d, 1H, J=16 Hz), 6.64 (s, 2H). Spectra of the natural compound were the same as those of the synthetic compound.

Synthesis of Sinapyl Alcohol[14] Acetylsinapic acid, prepared by acetylation of sinapic acid, was refluxed with thionyl cloride and the acid chloride was mixed with absolute ethanol to give an acethylsinapic acid ethyl ester. The ethyl ester was reacted with lithium aluminum hydride in an ice-salt-bath to product a salt-like substance. Upon treatment with

aqueous ammonium carbonate, this salt-like substance gave sinapyl alcohol. The product was isolated by column chromatography on Kieselgel 60. A white powder. ^{1}H-NMR ($CDCl_3$) δ: 3.88 (s, 6H), 4.30 (d, 2H, J=5.6 Hz), 5.69 (s, 1H), 6.23 (dt, 1H, J=16, 5.6 Hz), 6.50 (d, 1H, J=16 Hz), 6.61 (s, 2H). ^{13}C-NMR ($CDCl_3$): 56.2 (2C), 63.6 (1C), 103.2 (2C), 126.5 (1C), 128.1 (1C), 128.2 (1C), 131.2 (2C), 147.0 (1C). MS *m/z*: 210 (M^+), 182, 167, 149. UV$\lambda_{max}^{CH_3OH}$ nm: 274.

Coniferyl Alcohol A white amorphous solid. UV$\lambda_{max}^{CH_3OH}$ nm: 268. MS *m/z*: 180 (M^+), 137. ^{1}H-NMR ($CDCl_3$) δ: 3.91 (s, 3H), 4.30 (d, 2H, J=6 Hz), 5.62 (s, 1H), 6.23 (dt, 1H, J=16, 6 Hz), 6.54 (d, 1H, J=16 Hz), 6.86 (d, 1H, J=8 Hz), 6.9 (dd, 1H, J=8, 1.5 Hz), 6.92 (d, 1H, J=1.5 Hz). They are the same as those of standard compound.

Time Course Change of the Contents of HOAS and AS after Wounding in the Hairy Root Cultures of *A. belladonna* On the 10th day after the subculture of the hairy roots in MS solid medium, the hairy roots were subcultured in sealed petri dishes with 25 ml of MS liquid medium. On the 17th, 18th and 19th days, the hairy roots from three petri dishes were cut into 1—2 mm lengths and incubated in the same medium in which they had been cultured before wounding. On the 20th day, hairy roots were removed from the culture medium. The wounded and unwounded hairy roots (24 h, 48 h, 72 h), and the culture medium were analyzed separately. Each culture medium was accurately adjusted to 25 ml and an aliquot of 10 ml was passed through a rapid sample Sep Pak C-18 cartridge (TOYOPAK ODS M gel weight 300 mg). The cartridge was eluted with 3 ml of CH_3OH. The CH_3OH fraction was evaporated and resuspended in 1—1.5 ml of distilled water. The suspension was extracted four times with 1.5 ml of benzene. Benzene layer was evaporated to dryness and resuspended accurately in 1.5 ml of 30% CH_3OH. Each of the hairy roots was dried in vacuum at 45 °C for 8 h. It was weighted and homogenized using a mortar and a pestle. After being put into a tube with a cap, 10 ml of 70% CH_3OH was added and it was left for 24 h. Then, after extraction three times with 10 ml of 70% CH_3OH at 50 °C for 4 h, 70% CH_3OH was handled by the method described above.

Time Course Changes of Sinapyl and Coniferyl Alcohols in Hypocotyls of *P. sativum* after Wounding At 8 d after sowing, the hypocotyls were wounded by stabbing with a needle and cultivated continuously. Unwounded and wounded hypocotyls were obtained at 24 h, 48 h, 72 h. Three grams of each of hypocotyls was put in 25 ml of distilled water and homogenized with a waring blender for 1 min, then extracted by autoclaving (121 °C) for 10 min. Extract was adjusted to 25 ml with distilled water. Ten ml was passed through a rapid sample Sep Pak C-18 cartridge (TOYOPAK ODS M gel weight 300 mg). The cartridge was eluted with 5 ml of 90% CH_3OH. CH_3OH fraction was evaporated and resuspended in 1—1.5 ml of distilled water and extracted three times with 1.5 ml of benzene. Benzene layer was evaporated to dryness and resuspended accurately in 0.5 ml of 30% CH_3OH.

Acknowledgement The authors thank Prof. E. W. Nester of the University of Washington for his kind supply of *Agrobacterium* strain.

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15) Induction buffer is composed of AB salt, 2.5 mM KH_2PO_4, 3% sucrose and 20 mM morpholinoethane-sulfonic acid, pH 5.5.
16) LBMG liquid medium is an equal mixture of LB liquid medium (1% bactotryptone, 0.5% bacto yeast extract, and 1% NaCl) and MG liquid medium (1% mannitol, 0.232% sodium glutamate, 0.05% KH_2PO_4, 0.02% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.2% Biotin).
17) Z. Buffer is composed of 0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M 2-mercaptoethanol, pH 7.0.

Determination of the Ratio between Optical Isomers, Shikonin and Alkannin by High Performance Liquid Chromatography Analysis

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The optical isomers, shikonin and alkannin, can be separated by chiral phase high performance liquid chromatography. The results of analysis have shown that the commercial standards of "shikonin" or "alkannin" as well as the red pigments extracted from the roots of some Boraginaceous plants contain both optical isomers in various proportions.

Keywords Boraginaceae; naphthoquinone; shikonin; alkannin; stereoisomer; optical resolution; chiral phase HPLC; *Lithospermum erythrorhizon*; *Alkanna tinctoria*

Introduction

The roots of the Boraginaceous species, *Lithospermum erythrorhizon* SIEB. *et* ZUCC. and *Alkanna tinctoria* TAUSCH, have been used as crude drugs and red dyes since ancient times in the Far East and Europe, respectively.[1)] The former contains red naphthoquinone pigments, shikonin (**1**) and its esters,[2)] whereas the latter has an accumulation of the corresponding enantiomers, alkannin (**2**) and its esters.[2)] In many of the previous studies, however, the red naphthoquinone pigments were tentatively called shikonin or alkannin without examination of the optical activity, except for the determination of relative ratios between **1** and **2** in various Boraginaceous plants based on circular dichroism (CD) spectral data.[3)]

We now report a new and efficient method for determining the ratio of **1** to **2** in commercially available standards and root or callus extracts of Boraginaceous plants.

shikonin (**1**): $R^1=OH$, $R^2=H$
alkannin (**2**): $R^1=H$, $R^2=OH$

Chart 1

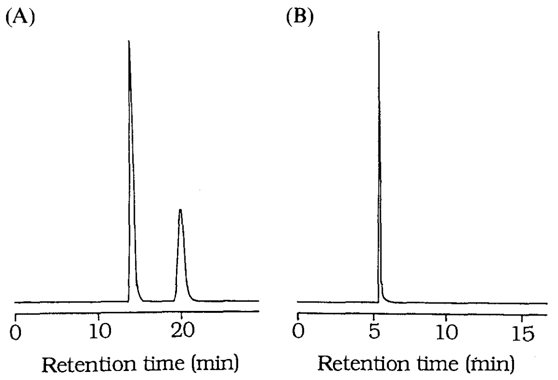

Fig. 1. High Performance Liquid Chromatograms of the Standard Sample No. 1 of "Shikonin" on Chiral Phase Column (A) and Reversed Phase Column (B)

Results and Discussion

A commercially available standard sample of "shikonin" showed two peaks on an analysis by high-performance liquid-chromatography (HPLC) with a chiral phase column (Chiralcel OD, Daicel Chemical Industries, Ltd.), whereas it showed only one peak by HPLC analysis using a reversed-phase column (Fig. 1).

Compounds corresponding to these two peaks were collected separately and shown to give identical data on nuclear magnetic resonance (^{1}H-NMR) spectra and negative secondary ion msss (SI-MS) spectra, except for CD spectral data; the former showed a positive maximum at 360 nm ($[\theta]=+7755$, $CHCl_3$), while the latter gave symmetrically inverted data. Therefore, the former was identified as shikonin (**1**) and the latter as alkannin (**2**).[3)]

The analytical method using HPLC with a chiral phase column was applied to determine the ratio between **1** and **2** in four commercial standard samples and total red naphthoquinone pigments extracted from seven different plant materials. As shown in Table I, all the commercial

TABLE I. Ratios between Shikonin and Alkannin in Commercially Available Standards

Samples	Shikonin : alkannin
Shikonin standard No. 1	62.0 : 38.0
No. 2	72.9 : 27.1
No. 3	16.6 : 83.4
Alkannin standard	93.1 : 6.9

TABLE II. Ratios between Shikonin and Alkannin in Total Red Naphthoquinone Pigments Obtained from Six Roots and One Callus Sample of Boraginaceous Plants

Plant	Shikonin : alkannin
Lithospermum erythrorhizon root[a)]	93.4 : 6.6
root[b)]	86.0 : 14.0
root[c)]	84.4 : 15.6
callus	83.6 : 16.4
Arnebia euchroma root[d)]	2.6 : 97.4
Alkanna tinctoria root[e)]	1.0 : 99.0
root[f)]	3.1 : 96.9

a) Cultivated in Kyoto, Japan. *b)* Cultivated in Hokkaido, Japan. *c)* "Ko-shikon" (Yingzicao) imported from China. *d)* "Nan-shikon" (Ruanzicao) imported from China. *e)* Collected in Turkey. *f)* Purchased in Greece.

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samples of shikonin or alkannin tested were neither pure nor racemic ("shikalkin"),[4] but were mixtures of the enantioisomers in different ratios. Table II shows that in the roots as well as in callus tissues of *Lithospermum erythrorhizon* the red pigment was composed of 84—93% shikonin and 7—16% alkannin. In contrast to *Lithospermum*, the red pigment of *Arnebia euchroma* (Nan-shikon, Ruanzicao) and *Alkanna tinctoria* was more than 95% alkannin. These results agree in general with those of the earlier studies.[3]

The analytical method employed in the present study would be useful not only for measuring the values of optical isomers but also for estimating the original species of crude drugs on the basis of the ratio of shikonin (**1**) to alkannin (**2**).

Experimental

NMR spectra were recorded on a Bruker AC-200 (200 MHz for ^{1}H-NMR) instrument, using tetramethylsilane as an internal standard. Negative secondary ion mass spectra were taken on a Hitachi M-2000 mass spectrometer. CD spectra were measured by a JASCO J-720 spectropolarimeter.

Standard Samples and Crude Drugs Three samples of shikonin standards were purchased from Wako Pure Chemical Industries, Ltd., Ichimaru Pharcos Co., Ltd. and Funakoshi Pharmaceutical Co., Ltd. and the alkannin standard was purchased from Funakoshi Pharmaceutical Co., Ltd. Root samples were obtained from *Lithospermum erythrorhizon* Sieb. *et* Zucc. cultivated in the field in Kyoto and Hokkaido, Japan. Callus tissues of *Lithospermum erythrorhizon* were cultured as described elsewhere.[3a] Crude drugs "Ko-shikon" (Chinese name: Yingzicao, the root of *Lithospermum erythrorhizon*) and "Nan-shikon" (Chinese name: Ruanzicao, the root of *Arnebia euchroma* Johnst.) were imported from China. Roots of *Alkanna tinctoria* Tausch were collected in Anatolia, Turkey in 1986 and purchased in Athens, Greece in 1990.

Preparation of Optically Pure Shikonin (1) and Alkannin (2) Optically pure **1** and **2** were prepared by separation of the shikonin standard No. 1 (Wako Pure Chemical Industries, Ltd.) by HPLC; column: Chiralcel OD (4.6 mm i.d. × 250 mm, Daicel Chemical Industries, Ltd.), mobile phase: hexane–2-propanol (90 : 10, v/v), pump: Waters M600 pump with Waters U6K injector, detector: Shimadzu SPD-6AV ultraviolet-visual detector (520 nm), integrator: Shimadzu C-R4A Chromatopack, flow rate: 0.75 ml/min and column temp.: room temperature. Retention time; shikonin (**1**): 14.44 min, alkannin (**2**): 20.54 min. Isomers **1** and **2** could not be separated but showed the same retention time (5.57 min) by reversed phase HPLC: column: Cosmosil 5C18-AR column (4.6 mm i.d. × 250 mm, Nacalai Tesque Inc.), mobile phase: CH_3CN–H_2O (65 : 35, v/v) containing trifluoroacetic acid (TFA), flow rate: 1.5 ml/min, the other conditions were same as those described above. The compounds were confirmed by ^{1}H-NMR, negative SI-MS, and CD spectral data.

Extraction and Analysis of 1 and 2 Each crude drug (0.2 g) was extracted with $CHCl_3$ (10 ml × 2) and the $CHCl_3$ layer was evaporated *in vacuo*. A solution of the $CHCl_3$ extract in 2-propanol (0.1 ml) was treated with 1 N NaOH (2 ml) for 6 h to hydrolyze **1** (or **2**) derivatives. The aqueous phase was adjusted to pH 3 with 1 N HCl and extracted with $CHCl_3$ (2 ml × 2). The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was subjected to chiral phase HPLC analysis to determine the absolute ratio of **1** to **2** on the basis of peak areas of shikonin and alkannin.

Effect of Alkaline Treatment on the Absolute Configuration of 1 and 2 The optical purity of **1** and **2** showed little change by the alkaline treatment described above, indicating that neither the absolute configuration of **1** nor that of **2** was changed to the opposite configuration by this treatment.

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Anti-inflammatory Effect of *Zingiber cassumunar* Roxb. and Its Active Principles

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The present study was carried out to elucidate the anti-inflammatory effect of the methanol extract obtained from the rhizomes of *Zingiber cassumunar* Roxb. and its active principles. The methanol extract was partitioned between ether and water, and then the ether-soluble fraction was extracted with *n*-hexane. The *n*-hexane-soluble fraction was chromatographed and part of the fraction was rechromatographed by silica gel column. Three compounds were isolated from the *n*-hexane-soluble fraction and the chemical structures of these compounds were identified as (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene, (*E*)-1-(3,4-dimethoxyphenyl)butadiene and zerumbone. The anti-inflammatory activity of these fractions was investigated on carrageenin-induced edema in rats, as well as on acetic acid-induced vascular permeability and writhing symptoms in mice. The methanol extract (*p.o.*) showed both anti-inflammatory activity and analgesic activity. These activities shifted successively to ether-soluble and *n*-hexane-soluble fractions and to (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene. These results suggest that the anti-inflammatory action and analgesic action of *Zingiber cassumunar* is the result of the (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene that it contains.

Keywords anti-inflammatory effect; carrageenin-induced edema; vascular permeability; *Zingiber cassumunar*; (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene

The rhizomes of *Zingiber cassumunar* Roxb. (*Z. cassumunar*) are used in Indonesian folk medicine for the treatment of colic, diarrhea, vermifuge, pain, rheumatism, as an analeptic for the uterus, *etc.*[1-5] We previously reported that the methanol extract obtained from the rhizomes of *Z. cassumunar* caused a lasting increase in bile secretion when orally administered to anesthetized rats, and that the cholagogic effect of the extract was attributable to the essential oil it contains.[6]

Although some pharmacological studies of *Z. cassumunar* have been reported,[7] there have been very few anti-inflammatory studies on this subject.

On the basis of these uses in folk medicine, the present study was carried out to elucidate the anti-inflammatory effect of a 70% methanol extract obtained from the rhizomes of *Z. cassumunar* and to identify the active principle(s).

Experimental

Materials Fresh rhizomes of *Z. cassumunar* were cultivated in the Bandung region and refluxed with 70% methanol three times for 6 h each time. The solution was filtered through filter paper and evaporated to give the extract under vacuum.

The extract was dissolved in ether and extracted with water three times. The ether phase was separated and evaporated to dryness under vacuum. The ether-soluble fraction was then dissolved in *n*-hexane and extracted with methanol three times. The *n*-hexane phase was separated and evaporated to dryness under vacuum.

As the *n*-hexane-soluble fraction showed three spots distinct from the methanol soluble fraction on thin layer chromatography (TLC) (Kieselgel 60 F_{254}, Merck), the fraction was chromatographed on a silica gel column, using an elution solvent of *n*-hexane and ethylacetate, to monitor these spots. The solvent system used was *n*-hexane : ethylacetate (50 : 1), and spots on the plate were detected under ultraviolet light. Three compounds were isolated from the *n*-hexane-soluble fraction and these compounds showed only a large spot on the thin layer plate, respectively. These compounds gave two oily compounds and one crystal as colorless needles. By using

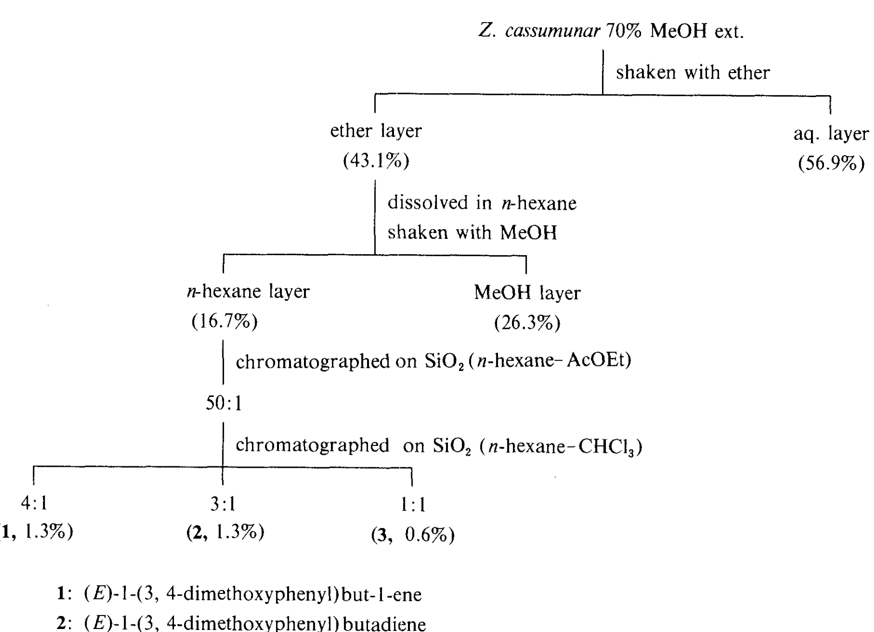

Fig. 1. Flow Diagram of Fractionation of the Methanol Extract Obtained from *Z. cassumunar*

Here, (%) indicates percentage yield calculated on the basis of the methanol extract obtained from *Z. cassumunar*.

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infrared (IR), ultraviolet (UV) and nuclear magnetic resonance (NMR) analyses, the chemical structures of these compounds were identical with (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene and (*E*)-1-(3,4-dimethoxyphenyl)-butadiene (two oily compounds), and zerumbone (one crystal), respectively. As shown in Fig. 1, the yields (%) were calculated on the basis of the methanol extract.

The methanol extract, each fraction, these compounds and indomethacin (Sigma) were each suspended in a 2% carboxymethyl cellulose (CMC) solution, and were administered orally 60—90 min before to assay for anti-inflammatory and analgesic effects. The dose for each of the fractions and the compounds was chosen based on the yields obtained from the 70% methanol extraction.

Carrageenin-Induced Hind-Paw Edema Test Male Wistar rats weighing 180—230 g were fasted for 16 h prior to the experiments, but were supplied with water *ad libitum*. Carrageenin (Picnin-A, Zushikagaku Lab., Inc.) was suspended in saline to make a 1% (w/v) suspension. The suspension of carrageenin (0.05 ml/animal) was injected subcutaneously into the right hind-paw 30 min after the test solutions had been administered.

The volume of the hind-paw was measured prior to administration of the test solutions by a water displacement transducer (LPU-0.1 A, Nihon Kohden). The hind-paw volumes were measured 30 min and 1 h after the suspension of carrageenin had been administered and then at intervals of 1 h for up to 6 h.

Control rats were treated similarly except that they received an oral dose of 2% CMC solution alone. The results were expressed as the percentage increase in hind-paw volume due to swelling, as compared with the initial hind-paw volume.

Acetic Acid-Induced Vascular Permeability Test Male ddY mice weighing 20—25 g were fasted for 2 h prior to experiments, but were supplied with water *ad libitum*. Four percent pontamine sky blue solution in saline (w/v) was injected intravenously into the tail vein 40 min after the administration of test solutions. After 30 min, a 1% acetic acid solution in saline (v/v) was injected intraperitoneally, and after 20 min, the mice were killed by dislocation of the neck and the abdominal wall was cut to expose the entrails. After washing of the entrails with saline, the washing were filtered through glass wool and collected in test tubes. To clear any turbidity due to protein, 0.1 ml of 1 N NaOH solution was added to each tube, and the absorbance was read at 590 nm in a spectrophotometer (model 200-10, Hitachi). Control mice were treated similarly, except that they received an oral dose of 2% CMC solution alone.

The vascular permeability effects were expressed in terms of the amount of total dye (μg/animal) which leaked into the intraperitoneal cavity.

Acetic Acid-Induced Writhing Test Male ddY mice weighing 20—25 g were fasted for 2 h, but were supplied with water *ad libitum*. A 0.7% solution of acetic acid in saline (v/v) was injected intraperitoneally 85 min after the test solutions had been administered. After 5 min, the number of writhes induced by the acetic acid solution was counted for 10 min.

Control mice were treated similarly, except that they received an oral dose of 2% CMC solution alone.

Statistical Analysis Data were expressed as the mean value ± standard error. All results were analyzed for variance by Bartlett's method, and significant differences were subsequently examined by Duncan's method.

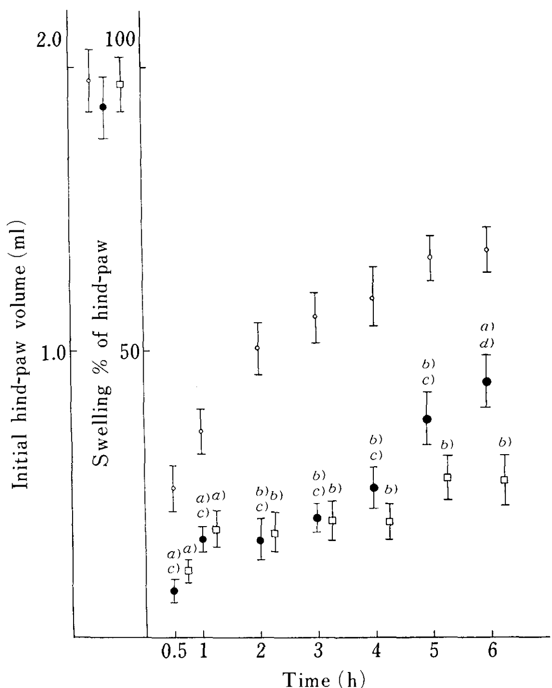

Fig. 2. Effect of the Methanol Extract and Indomethacin on the Paw Edema Induced by Carrageenin in Rats

The results were expressed as the percentage increase in hind-paw volume due to swelling (%) (right column), compared with the initial hind-paw volume (ml) before carrageenin injection (left column). *a, b*) Significantly different from the control at $p<0.01$ and $p<0.001$, respectively. *c*) Not significantly different from indomethacin and *d*) significantly different from indomethacin at $p<0.05$. ○, control (2% CMC) (*p.o.*) ($N=7$); ●, *Z. cassumunar* 3.0 g/kg ($N=7$); □, indomethacin 10 mg/kg ($N=7$).

Table I. Effect of the Methanol Extract and Indomethacin on the Increased Vascular Permeability Induced by Acetic Acid in Mice

Compounds	Dose (g/kg *p.o.*)	No. of animals	Amount of leaked dye (μg/animal)
Control (2% CMC)		10	395.2 ± 37.1
Z. cassumunar	0.3	8	304.3 ± 11.4$^{a,b)}$
	1	8	228.0 ± 10.9$^{c,d)}$
Indomethacin	0.01	8	219.8 ± 26.3$^{c)}$

a, c) Significantly different from the control at $p<0.05$ and $p<0.01$, respectively. *b*) Significantly different from indomethacin at $p<0.05$ and *d*) not significantly different from indomethacin.

Table II. Analgesic Effect of the Methanol Extract and Indomethacin on the Writhing Symptom Induced by Acetic Acid in Mice

Compounds	Dose (g/kg *p.o.*)	No. of animals	No. of writhes (in 10 min)
Control (2% CMC)		10	39.2 ± 1.9
Z. cassumunar	1	7	37.3 ± 3.2
	3	8	21.0 ± 3.1$^{a,b)}$
Indomethacin	0.1	9	22.3 ± 4.0$^{c)}$

a, c) Significantly different from the control at $p<0.01$ and $p<0.001$, respectively. *b*) Not significantly different from indomethacin.

Results

Effect of Methanol Extract Obtained from *Z. cassumunar* The methanol extract (at 3 g/kg) showed a lasting inhibition of the edema induced by carrageenin during the 6 h period. The inhibitory potency was about the same as that of indomethacin (at 10 mg/kg) (Fig. 2). In the preliminary experiment, the lower dose of methanol extract (at 1 g/kg) did not show a significant inhibitory effect on the edema (data not shown in the figure).

The same doses of methanol extract also reduced dose-dependently the number of writhes induced by acetic acid. The inhibitory potency induced by the extract (at 3 g/kg) was about the same as that of indomethacin (at 10 mg/kg) (Table I).

The methanol extract (at doses of 0.3 and 1 g/kg) inhibited dose-dependently the increase of dye leakage induced by acetic acid. Indomethacin (at 10 mg/kg) inhibited the dye leakage with a potency about the same as that of the methanol extract (at 1 g/kg) (Table II).

Effects of Each Fraction Obtained from the Methanol Extract The ether-soluble fraction obtained from the

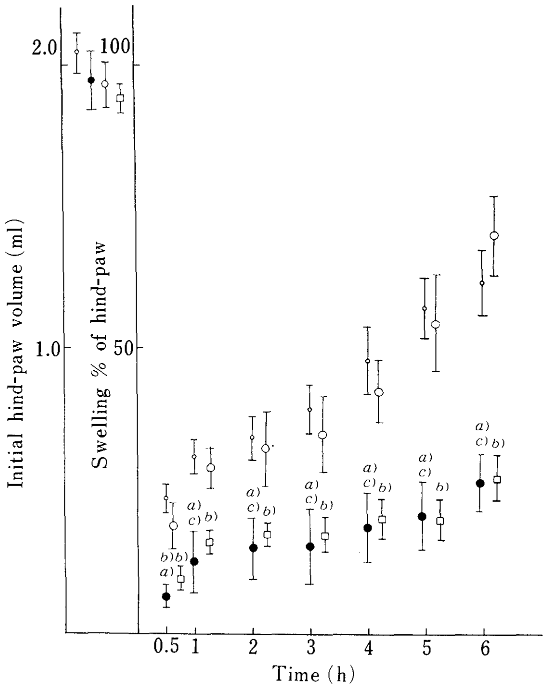

Fig. 3. Effect of the Ether-Soluble Fraction, the Water-Soluble Fraction and Indomethacin on the Paw Edema Induced by Carrageenin in Rats

The results were expressed as the percentage increase in hind-paw volume due to swelling (%) (right column), compared with the initial hind-paw volume (ml) before carrageenin injection (left column). *a*, *b*) Significantly different from the control at $p<0.01$ and $p<0.001$, respectively. *c*) Not significantly different from indomethacin. ○, control (2% CMC) (*p.o.*) ($N=11$); ●, *Z. cassumunar* ether layer 1.3 g/kg ($N=7$); ◯, *Z. cassumunar* aq. layer 1.7 g/kg ($N=7$); □, indomethacin 10 mg/kg ($N=11$).

Table III. Effect of the Ether-Soluble Fraction, the Water-Soluble Fraction and Indomethacin on the Increased Vascular Permeability Induced by Acetic Acid in Mice

Compounds	Dose (g/kg *p.o.*)	No. of animals	Amount of leaked dye (μg/animal)
Control (2% CMC)		10	392.5±14.9
Z. cassumunar ether layer	0.5	8	205.0±14.6[a,b]
aq. layer	0.6	8	384.9±24.7
Indomethacin	0.01	8	205.4±17.6[a]

a) Significantly different from the control at $p<0.001$. *b*) Not significantly different from indomethacin.

methanol extract (at 1.3 g/kg) showed a lasting inhibition of edema beginning from 30 min until 6 h after injection of carrageenin. On the other hand, the water-soluble fraction (at 1.7 g/kg) did not inhibit the edema. The inhibitory potency induced by the ether-soluble fraction was about the same as that of indomethacin (at 10 mg/kg) (Fig. 3).

The ether-soluble fraction (at 0.5 g/kg) inhibited the increase of dye leakage induced by acetic acid, while the water-soluble fraction (at 0.6 g/kg) did not. The inhibitory potency induced by the ether soluble fraction was about the same as that of indomethacin (at 10 mg/kg) (Table III).

The *n*-hexane-soluble fraction obtained from the ether-soluble fraction (at 0.2 g/kg) inhibited the increase of dye leakage induced by acetic acid, while the methanol-soluble fraction (at 0.3 g/kg) did not. The inhibitory potency induced by the *n*-hexane-soluble fraction was about the same as that of indomethacin (at 10 mg/kg) (Table IV).

Table IV. Effect of the *n*-Hexane-Soluble Fraction, the Methanol-Soluble Fraction and Indomethacin on the Increased Vascular Permeability Induced by Acetic Acid in Mice

Compounds	Dose (g/kg *p.o.*)	No. of animals	Amount of leaked dye (μg/animal)
Control (2% CMC)		16	439.2±19.2
Z. cassumunar *n*-hexane layer	0.2	8	300.5±23.8[a,b]
MeOH layer	0.3	8	415.9±19.2
Indomethacin	0.01	14	274.9±25.8[a]

a) Significantly different from the control at $p<0.001$. *b*) Not significantly different from indomethacin.

Table V. Effect of (*E*)-1-(3,4-Dimethoxyphenyl)but-1-ene, (*E*)-1-(3,4-Dimethoxyphenyl)butadiene and Zerumbone from the *n*-Hexane-Soluble Fraction and Indomethacin on the Increased Vascular Permeability and Writhing Induced by Acetic Acid in Mice

Compounds	Dose (g/kg *p.o.*)	No. of animals	Amount of leaked dye (μg/animal)	No. of writhes (in 10 min)
Control (2% CMC)		6	245.1±23.0	46.3±6.2
(*E*)-1-(3,4-Dimethoxy-phenyl)but-1-ene	0.016	6	130.0±28.1[c,b]	31.2±3.1[a,d]
(*E*)-1-(3,4-Dimethoxy-phenyl)butadiene	0.016	6	224.7±28.4	45.3±1.2
Zerumbone	0.008	6	269.0±19.1	50.0±3.8
Indomethacin	0.01	6	136.5±27.8[c]	20.2±4.2[c]

a, *c*) Significantly different from the control at $p<0.05$ and $p<0.01$, respectively. *b*, *d*) Not significantly different from indomethacin, respectively.

From these results, it was suggested that the pharmacologically active principle(s) passed into the *n*-hexane-soluble fraction.

As shown in Table V, among the compounds isolated from the *n*-hexane-soluble fraction, only (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene (at 0.016 g/kg) inhibited the increase in dye leakage and it also reduced the number of writhes induced by acetic acid. On the other hand, (*E*)-1-(3,4-dimethoxyphenyl)butadiene (at 0.016 g/kg) and zerumbone (at 0.008 g/kg) did not significantly inhibit the increase of dye leakage and neither compound reduced the number of writhes induced by acetic acid.

The inhibitory potency induced by (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene was about the same as that of indomethacin (at 10 mg/kg).

Discussion

In the present study, it was found that the 70% methanol extract obtained from *Z. cassumunar* significantly inhibited edema induced by carrageenin (at 3 g/kg), the increase in dye leakage induced by acetic acid (at 1 g/kg) and the number of writhes induced by acetic acid (at 3 g/kg). It was also found that the inhibitory potency induced by the extract was about the same as that of indomethacin at 10 mg/kg.

It is well known that irritating compounds sometimes cause pseudo inhibition of the edema induced by carrageenin. But the methanol extract also inhibited the increase in dye leakage induced by acetic acid and the number of writhes induced by acetic acid.

From these results, it seems likely that the methanol extract does not have an irritating effect, but both anti-inflammatory and analgesic effects. Therefore, it was considered

worthwhile to elucidate the anti-inflammatory activity of the extract and to isolate its active principles.

The ether-soluble fraction obtained from the methanol extract inhibited both edema induced by carrageenin and the increase in dye leakage induced by acetic acid, but the water-soluble fraction did not. As the anti-inflammatory activity had been concentrated in the ether-soluble fraction, this was further fractionated, based on the results of an anti-inflammatory activity assay using the experimental model of dye leakage induced by acetic acid.

The activity shifted successively to the *n*-hexane-soluble fraction. Since the potency of the inhibitory effects induced by these fractions was approximately the same as that of a fixed dose of indomethacin in all experiments, it is considered that the anti-inflammatory activity had been almost entirely in the *n*-hexane-soluble fraction. Then, the active principle was isolated from this fraction and its chemical structure was identified as (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene.

(*E*)-1-(3,4-dimethoxyphenyl)but-1-ene inhibited the increase in dye leakage induced by acetic acid and also reduced the number of writhes induced by acetic acid.

These results suggest that the anti-inflammatory effect of the methanol extract is due to its (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene content and also that it may exert an analgesic effect.

It is very interesting that (*E*)-1-(3,4-dimethoxyphenyl)-but-1-ene showed an anti-inflammatory effect, but (*E*)-1-(3,4-dimethoxyphenyl)butadiene did not, suggesting the butene moiety in their chemical structure is important in producing the anti-inflammatory effect.

Kuroyanagi *et al.* and Tuntiwachwuttikul *et al.* reported that (*E*)-1-(3,4-dimetoxyphenyl)but-1-ene and (*E*)-1-(3,4-dimethoxyphenyl)butadiene were isolated from a chloroform-soluble or hexane-soluble extraction of the rhizome of *Z. cassumunar*, respectively.[8,9] And, there were some reports in which zerumbone was isolated from the rhizome of *Zingiber zerumbet* (L.) Sm., but there have been few reports in which it was isolated from the rhizome of *Z. cassumunar*.[10,11]

Kanjanapothi *et al.* reported that (*E*)-4-(3,4-dimethoxyphenyl)but-3-ene-1-ol was isolated from a hexane-soluble extract of *Z. cassumunar* and that it exhibited a dose-dependent relaxant effect on the uterus of nonpregnant rats, and also that it may act by mechanisms similar to that of papaverine.[7] But, there have been few reports about the pharmacological effects of (*E*)-4-(3,4-dimethoxyphenyl)but-1-ene, especially anti-inflammatory and analgesic effects.

It is well known that the development of edema induced by carrageenin and the increase in vascular permeability induced by acetic acid correspond to the early exudative stage of inflammation, one of the important processes of inflammatory pathology.[12,13] (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene inhibited the edema induced by carrageenin and the increase of the vascular permeability induced by acetic acid in the present study, which shows that it exerts an anti-inflammatory effect at an early exudative stage of inflammation.

Brown *et al.* then reported that many centrally acting drugs inhibited the edema induced by carrageenin in the hind-paw of rats.[14]

Although neither the fractions nor the (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene, at the doses used in this experiment (0.016 g/kg), was found to have any apparent effect on the central nervous system or on toxicity in mice and rats, these reports suggest that the anti-inflammatory effects of the compound may be partly exerted through the central nervous system.

Acknowledgments The author is indebted to Dr. S. Soedigdo, Professor at the Institute Technology of Bandung, for collection of the rhizomes of *Z. cassumunar*. This work was supported in part by a grant from the Special Coordinating Funds for Promoting Science and Technology (1984–1987) of the Science and Technology Agency of Japan.

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Studies on Absorption, Distribution, Excretion and Metabolism of Ginseng Saponins. VII.[1] Comparison of the Decomposition Modes of Ginsenoside-Rb_1 and -Rb_2 in the Digestive Tract of Rats

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In order to clarify some similarities and differences of decomposition modes between 20(*S*)-protopanaxadiol (20(*S*)-ppd) saponins, represented by ginsenoside Rb_1 (Rb_1) and ginsenoside Rb_2 (Rb_2), the decompositions of Rb_1 and Rb_2 in the rat gastrointestinal tract, 0.1 N HCl and crude hesperidinase were investigated in detail. As in the case of Rb_2 reported previously,[1] Rb_1 was hydrolyzed to 20(*R,S*)-ginsenoside Rg_3 in 0.1 N HCl. On the other hand, hydroperoxidation of Rb_1 occurred in rat stomach; the major hydroperoxide was separated and identified as the 25-hydroperoxy-23-ene derivative of Rb_1 (VIII) by ^1H- and ^{13}C-nuclear magnetic resonance and fast atom bombardment mass spectrometry. The decomposition modes of 20(*S*)-ppd saponins (Rb_1 and Rb_2) differed from that of 20(*S*)-protopanaxatriol saponin (Rg_1) in rat stomach. In rat large intestine, five decomposition products of Rb_1 were observed by thin-layer chromatography, and these were identified as gypenoside XVII (G-XVII), ginsenoside Rd (Rd), ginsenoside F_2 (F_2), compound K (C-K) and VIII. The decomposition modes of Rb_1 and Rb_2, both 20(*S*)-ppd saponins, are considered to be different because of the hydrolysis rate in the terminal sugar moiety at the C-20 hydroxyl group in the rat large intestine. Using crude hesperidinase, Rb_1 was decomposed to G-XVII, F_2 and C-K, and Rb_2 was decomposed to 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinopyranosyl(1→6)-β-D-glucopyranosyl]-20(*S*)-ppd, F_2 and C-K. Consequently, it appears that hydrolysis by β-glucosidase, which is present in the rat large intestine, is distinct from that by crude hesperidinase. Tetracycline-resistant bacteria decomposed both Rb_1 and Rb_2 to their respective prosapogenins, except for Rd, and their respective hydroperoxides, while Rd and hydroperoxides of Rb_1 and Rb_2 were produced by enteric enzymes.

Keywords ginsenoside Rb_1; ginsenoside Rb_2; decomposition mode; rat gastrointestinal tract; hydroperoxide; ^{13}C-NMR; FAB-MS; ginsenoside prosapogenin; TLC; HPLC

We have been studying the pharmacokinetics of the root of *Panax ginseng* C. A. MEYER (Araliaceae), including features such as absorption, distribution, excretion and metabolism of ginsenosides, which are regarded as the components principally responsible for the pharmacological activities of Ginseng Radix. Initially, we studied and reported the pharmacokinetics of ginsenoside Rg_1 (Rg_1), one of the 20(*S*)-protopanaxatriol (20(*S*)-ppt) saponins, and ginsenoside Rb_1 (Rb_1), one of the 20(*S*)-protopanaxadiol (20(*S*)-ppd) saponins, in the rat gastrointestinal tract.[2] It was found that (1) Rg_1 was decomposed mainly to 20(*R,S*)-ginsenoside Rh_1 (Rh_1), their 25-hydrated derivatives (I and II) and ginsenoside F_1 (F_1) by rat gastric juice and enteric bacteria, whereas (2) Rb_1 was only slightly decomposed by rat gastric juice, and decomposed mainly to ginsenoside Rd (Rd) by rat enteric enzymes. These findings suggested that 20(*S*)-ppt saponin and 20(*S*)-ppd saponin had obviously different decomposition modes. We then studied the pharmacokinetics of ginsenoside Rb_2 (Rb_2), one of the 20(*S*)-ppd saponins, and isolated and identified five prosapogenins of Rb_2: Rd, 3-*O*-β-D-glucopyranosyl-20-*O*-[α-L-arabinopyranosyl(1→6)-β-D-glucopyranosyl]-20(*S*)-ppd (III), ginsenoside-F_2 (F_2), 20-*O*-[α-L-arabinopyranosyl(1→6)-β-D-glucopyranosyl]-20(*S*)-ppd (IV) and compound K (C-K), as metabolites in the rat gastrointestinal tract.[3] We also found that Rb_2 was changed to various hydroperoxides, mainly 3β,12β,20(*S*)-trihydroxy-25-hydroperoxydammar-23-en 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranoside]-20-*O*-[α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside] (V), in the rat stomach and large intestine.[1] The decomposition mode of Rb_2 has not yet been investigated in detail, however, we earlier reported that Rg_1 easily undergoes hydrolysis of the C-20 glycosyl moiety and hydration of the side-chain double bond.[2] However, we have not yet investigated the behavior of Rb_1 in the rat gastrointestinal tract in any detail. The aim of this study, therefore, was to clarify similarities and differences in the decomposition modes between 20(*S*)-ppt saponins and 20(*S*)-ppd saponins, and between 20(*S*)-ppd saponins as represented by Rb_1 and Rb_2 in the rat gastrointestinal tract.

Experimental

Materials and Equipment Most of the materials and equipment were the same as described in our previous paper.[1] Rb_1 was supplied by the Japan-Korea Red Ginseng Co., Ltd. Crude hesperidinase was kindly supplied by Tanabe Pharmaceutical Co., Ltd. Tetracycline hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. ^{13}C-Nuclear magnetic resonance (^{13}C-NMR) spectra were measured with a JEOL model GSX-500 (125.6 MHz) spectrometer.

Biological Decomposition of Rb_1 and Rb_2 1) Decomposition of Rb_1 in Rat Stomach and Large Intestine: Rb_1 (100 mg/kg, 2% aqueous solution) was administered orally to rats. After exsanguination from the abdominal artery under ether anesthesia at 30 or 60 min after administration, the stomach and large intestine were removed. The gut contents were flushed with MeOH and treated according to the procedure in Chart 1. In rat stomach, Rb_1 was found to be only slightly decomposed, and a small quantity of a decomposition product with an *Rf* value lower than that of Rb_1 was detected by normal-phase thin-layer chromatography (TLC). In rat large intestine, on the other hand, five decomposition products were observed by normal-phase TLC, and the products with higher *Rf* values than Rb_1 were identified as gypenoside XVII (G-XVII), Rd, ginsenoside F_2 (F_2) and C-K by comparison of their TLC and high performance liquid chromatography (HPLC) behaviors with those of authentic samples. Also, a spot identical to the decomposition product from rat stomach was detected in rat large intestine. Therefore, Rb_1 (200 mg) was incubated at 37 °C for 1 h with rat cecal contents in 100 ml of 0.9% saline. MeOH (400 ml) was added, and the mixture was centrifuged (3000 rpm, 10 min). The supernatant was evaporated to dryness below 40 °C under reduced pressure, and the residue was subjected to preparative HPLC (32% aqueous CH_3CN), yielding VI (1 mg), VII (0.6 mg), VIII (7.5 mg), IX (2 mg) and Rb_1 (50 mg). VIII was identified from fast atom bombardment mass spectra (FAB-MS) data and by comparison of their ^{13}C-

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NMR spectral data with reported values.[1] No ^{13}C-NMR spectra were obtained for VI, VII and IX because of their low yield, but FAB-MS data for these compounds were measured.

Rb_1: FAB-MS *m/z*: 1109 $(M+H)^+$, 1131 $(M+Na)^+$. ^1H-NMR (pyridine-d_5) δ: 0.82, 0.95, 0.96, 1.10, 1.27, 1.64 (3H each, all s, *tert*-$CH_3 \times 6$), 1.61, 1.65 (3H each, both s, vinyl. CH_3), 4.89, 5.06, 5.10, 5.33 (1H each, all d, J=7.6, 7.6, 7.6, 7.6 Hz, respectively, anomeric H $\times$ 4), 5.31 (1H, t, J=6.3 Hz, C_{24}-H). ^{13}C-NMR: shown in Table I.

VIII: FAB-MS *m/z*: 1163 $(M+Na)^+$. ^1H-NMR (pyridine-d_5) δ: 0.87, 0.90, 1.04, 1.11, 1.30, 1.58, 1.59, 1.61 (3H each, all s, *tert*-$CH_3 \times 8$), 4.90, 5.08, 5.16, 5.34 (1H each, all d, J=7.3, 7.9, 7.9, 7.6 Hz, respectively, anomeric H $\times$ 4), 6.13 (2H, s, C_{23}-H and C_{24}-H). ^{13}C-NMR: shown in Table I.

VI: FAB-MS *m/z*: 1125 $(M+H)^+$, 1147 $(M+Na)^+$.

VII: FAB-MS *m/z*: 1125 $(M+H)^+$, 1147 $(M+Na)^+$.

IX: FAB-MS *m/z*: 1141 $(M+H)^+$, 1163 $(M+Na)^+$.

2) Time Courses of Decomposition of Rb_1 and Rb_2 by Rat Cecal Contents *in Vitro*: The whole cecal contents of two normal rats were suspended in 0.9% saline (80 ml) and divided into two parts. Rb_1 (20 mg) and Rb_2 (20 mg) were added to each suspension of rat cecal contents, and incubated at 37 °C. After 0.5, 1, 3 and 6 h, a 10-ml sample was removed from each incubation mixture and treated according to the procedure shown in Chart 1.

3) Decomposition Modes of Rb_1 and Rb_2 by Rat Cecal Contents *in Vitro*: The whole cecal contents of two normal rats were suspended in 0.9% saline (100 ml) and treated according to the procedure shown in Chart 3, yielding media (I—IV) for incubation. Rb_1 or Rb_2 (5 mg) was added to 10 ml of each medium and incubated at 37 °C. After 1 h incubation of Rb_1 (3 h in the case of Rb_2), each incubation mixture was treated according to the procedure shown in Chart 1.

Chemical and Enzymatic Decomposition of Rb_1 and Rb_2 1) Hydrolysis of Rb_1 with 0.1 N HCl: Rb_1 (10 mg) was dissolved in 2 ml of 0.1 N HCl, and incubated at 37 °C for 4 h, then treated with a Sep-Pak® C_{18} cartridge (Sep-Pak) according to the procedure shown in Chart 1. The obtained decomposition products were subjected to TLC and HPLC, and identified

rat stomach or large intestine content
incubation mixture with rat cecal content → added MeOH
centrifuged at 3000 rpm for 10 min
supernatant | precipitate
evaporated to dryness *in vacuo*
residue
suspended in H_2O
incubation mixture with 0.1 N HCl or crude hesperidinase → treated with a Sep-Pak C_{18} cartridge washed with H_2O, 40% aqueous MeOH, eluted with MeOH
eluate
evaporated to dryness *in vacuo*
decomposition products
TLC and HPLC

Chart 1. Procedure for Obtaining Decomposition Products

Table I. ^{13}C-NMR Data for Rb_1, VIII, Rb_2, and V[a]

Carbon	Rb_1	VIII	Rb_2	V
C-17	51.7	52.1	51.7	52.0
C-20	83.5	83.3	83.5	83.3
C-21	22.5	23.5	22.4	23.4
C-22	36.2	40.1	36.2	40.2
C-23	23.6	126.6	23.2	126.6
C-24	126.0	138.1	126.0	138.1
C-25	131.1	81.3	131.1	81.3
C-26	25.8	25.5	25.8	25.4
C-27	18.0	25.1	17.9	25.1
3-*O*-β-D-Glucopyranosyl				
C-1′	105.1	105.1	105.1	105.1
2′-*O*-β-D-Glucopyranosyl				
C-1″	106.1	106.1	106.1	106.1
20-*O*-β-D-Glucopyranosyl				
C-1‴	98.1	98.1	98.1	98.3
6‴-*O*-β-D-Glucopyranosyl				
C-1⁗	105.4	105.4		
6‴-*O*-α-L-Arabinopyranosyl				
C-1⁗			104.6	104.3

a) Rb_1 and VIII were measured at 125.6 MHz, and Rb_2 and V at 67.8 MHz.

	R^1	R^2	R^3	R^4
Rb_1	–O–glc^2–glc	–O–glc^6–glc	–CH_3	–H
Rb_2	–O–glc^2–glc	–O–glc^6–arap	–CH_3	–H
Rg_1	–OH	–O–glc	–CH_3	–O–glc
20(*S*)-Rg_3	–O–glc^2–glc	–OH	–CH_3	–H
Rg_3	–O–glc^2–glc	–CH_3	–OH	–H
Rd	–O–glc^2–glc	–O–glc	–CH_3	–H
G-XVII	–O–glc	–O–glc^6–glc	–CH_3	–H
III	–O–glc	–O–glc^6–arap	–CH_3	–H
F_2	–O–glc	–O–glc	–CH_3	–H
IV	–OH	–O–glc^6–arap	–CH_3	–H
C-K	–OH	–O–glc	–CH_3	–H

	R^1	R^2
VIII	–O–glc^2–glc	–O–glc^6–glc
V	–O–glc^2–glc	–O–glc^6–arap

Chart 2. Chemical Structures

glc, β-D-glucopyranosyl; arap, α-L-arabinopyranosyl.

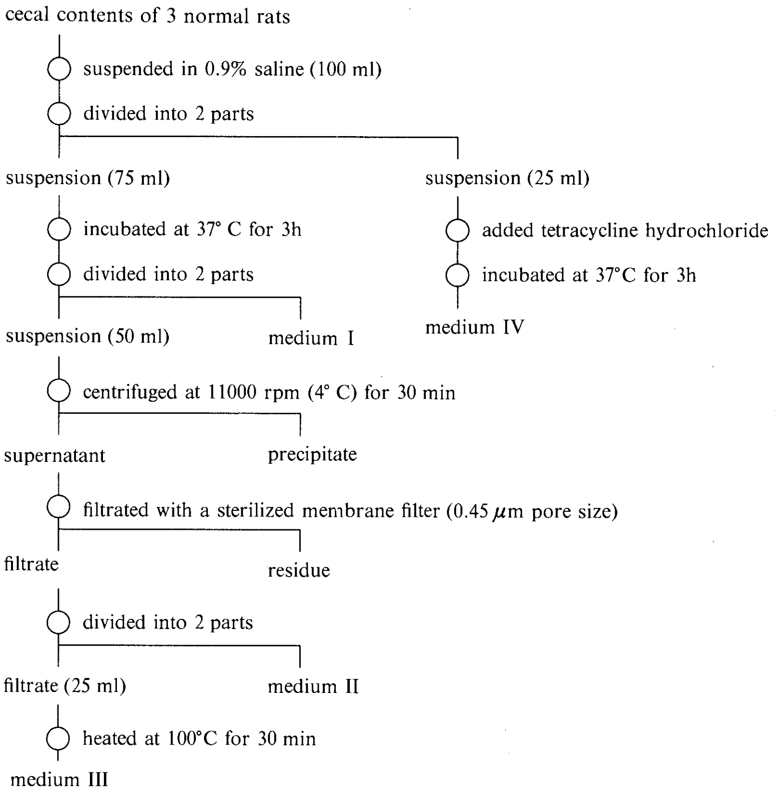

Chart 3. Procedure for Preparation of Incubation Media of Rat Cecal Contents

as 20(*S*)-ginsenoside Rg_3 (20(*S*)-Rg_3) and ginsenoside Rg_3 (Rg_3) by comparison of their TLC and HPLC behavior with that of authentic samples.

2) Enzymatic Decomposition of Rb_1 and Rb_2 by Crude Hesperidinase: Rb_1 or Rb_2 (10 mg) was dissolved in 5 ml of 0.2 M Na_2HPO_4–0.1 M citric acid buffer (pH 5.0), and crude hesperidinase (5 mg) was added. After incubation at 37 °C for 24 h, the mixture was treated according to the procedure shown in Chart 1. Decomposition products of Rb_1 were identified as G-XVII, F_2 and C-K, and those of Rb_2 as III, F_2 and C-K by comparison of their TLC and HPLC behavior with that of authentic samples.

Results and Discussion

Odani and his colleagues reported that Rb_1 was decomposed mainly to Rd in the rat large intestine after oral administration, but they did not investigate in detail other decomposed products in the rat gastrointestinal tract.[2] In the present study, therefore, the decomposition products of Rb_1 were further investigated in the digestive tract of the rat. As in the previous study,[2] we also noticed that Rb_1 was only a little decomposed in rat stomach, and that a small quantity of Rb_1 was changed into a substance with an *Rf* value lower than that of Rb_1 on normal-phase TLC (Fig. 1), and which was detected among the decomposition products of Rb_1 in the rat large intestine. This was similar to the case for Rb_2 reported previously,[1] suggesting that hydroperoxidation of Rb_1 might occur in rat stomach. By reference to data from our previous study,[1] we succeeded in isolating four products (designated VI, VII, VIII and IX) derived from Rb_1 by incubation with rat cecal contents. Comparison of their $(M+Na)^+$ ions by FAB-MS with those of Rb_1 led us to speculate that a hydroxyl group was present in VI and VII, and that a hydroperoxyl group existed in VIII and IX. By comparison of the ^{13}C-NMR data for VIII with those for V, we determined VIII to be 3*β*,12*β*,20(*S*)-trihydroxy-25-hydroperoxydammar-23-en 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranoside]-20-*O*-[*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranoside]. Although VI, VII and IX could not be

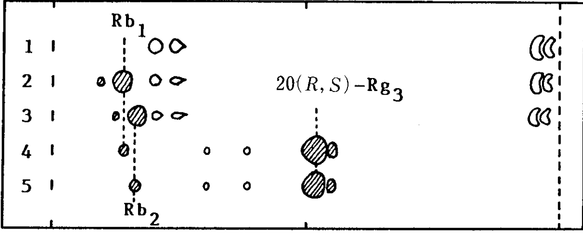

Fig. 1. Thin-Layer Chromatogram of Decomposition Products of Rb_1 and Rb_2 in Rat Stomach or in 0.1 N HCl

Developing solvent, $CHCl_3$–MeOH–H_2O (65 : 35 : 10, v/v, lower phase); plate, precoated Silica gel 60 F_{254} (Merck); detecting reagent, 1% of $Ce(SO_4)_2$–10% H_2SO_4 solution, with heating at 150 °C for 4 min. 1, normal rat; 2, Rb_1 (100 mg/kg, *p.o.*)-administered rat (1 h after treatment); 3, Rb_2 (100 mg/kg, *p.o.*)-administered rat (1.5 h after treatment); 4, Rb_1 in 0.1 N HCl; 5, Rb_2 in 0.1 N HCl.

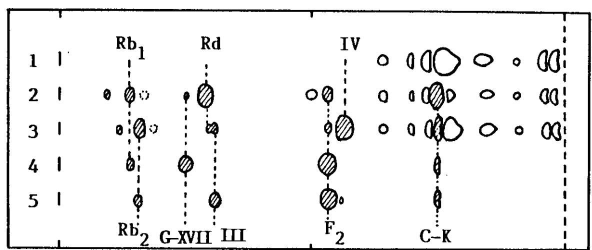

Fig. 2. Thin-Layer Chromatogram of Decomposition Products of Rb_1 and Rb_2 in Rat Large Intestine or by Crude Hesperidinase

TLC conditions were the same as those described in Fig. 1. 1, normal rat; 2, Rb_1 (100 mg/kg, *p.o.*)-administered rat (3 h after treatment); 3, Rb_2 (100 mg/kg, *p.o.*)-administered rat (6 h after treatment); 4, Rb_1 by crude hesperidinase; 5, Rb_2 by crude hesperidinasein.

TABLE II. Time Courses of Decomposition of Rb_1 and Rb_2 by Rat Cecal Contents (*in Vitro*)[a]

Incubation time (h)	Decomposition products of Rb_1						Decomposition products of Rb_2						
	Rb_1	VIII	G-XVII	Rd	F_2	C-K	Rb_2	V	Rd	III	F_2	IV	C-K
0.5	+	+	+	++	±	±	++	±	±	+	−	±	−
1	±	+	±	+	+	+	+	+	+	++	±	+	+
3	−	−	−	−	−	++	±	+	±	+	+	++	+
6	−	−	−	−	−	++	−	±	−	−	−	±	++

a) (+) and (−) indicate detectable and undetectable, respectively. (++) indicates the major product.

TABLE III. The Decomposition Modes of Rb_1 and Rb_2 by Rat Cecal Contents[a]

Treatment[b] (*in Vitro*)	Decomposition products of Rb_1[c]					Decomposition products of Rb_2[c]					
	VIII	G-XVII	Rd	F_2	C-K	V	Rd	III	F_2	IV	C-K
Medium I	+	±	++	+	+	+	±	+	+	++	±
Medium II	+	±	+	−	−	+	+	−	−	−	−
Medium III	−	−	−	−	−	−	−	−	−	−	−
Medium IV	+	±	+	+	+	+	±	+	±	++	±

a) (+) and (−) indicate detectable and undetectable, respectively. (++) indicates the major product. *b*) Medium I, normal rat; medium II, sterile filtrate; medium III, heated sterile filtrate; medium IV, tetracycline treated normal rat. *c*) Rb_1 was incubated for 1 h, and Rb_2 was incubated for 3 h.

determined by ^{13}C-NMR because of their small yield, we assumed that they were likely to be the 25-hydroxy-23-en (VI), 24-hydroxy-25-en (VII) and 24-hydroperoxy-25-en (IX) derivatives of Rb_1, as in the case of Rb_2. It thus was revealed that hydroperoxidation of Rb_1 occurred in the rat stomach, similar to the case of Rb_2.

Next, we investigated the effect of treating Rb_1 with 0.1 N HCl, with an acidity similar to that of gastric juice. As shown in Fig. 1, the result was same as that for Rb_2. The major decomposition products were 20(*R*,*S*)-Rg_3, quite different from the four products in rat stomach. The above data showed more clearly that 20(*S*)-ppd saponins (Rb_1 and Rb_2) undergo partial hydroperoxidation in rat stomach, whereas they are easily hydrolyzed by 0.1 N HCl, and that the decomposition mode of 20(*S*)-ppd saponins is different from that of 20(*S*)-ppt saponin (Rg_1), which easily undergoes both hydrolysis and hydration in rat stomach and 0.1 N HCl.

As shown in Fig. 2, five decomposition products were observed by TLC in a sample of rat large intestine after oral administration of Rb_1 and incubation of Rb_1 with rat cecal contents. These were identified as G-XVII, Rd, F_2, C-K and VIII, as described in the Experimental section. The formation of these prosapogenins of Rb_1 revealed that the decomposition pathways of Rb_1 and Rb_2 are similar, *i.e.* decomposition begins with cleavage of the terminal sugar moiety at the C-3 or C-20 hydroxyl group, and the reaction proceeds *via* stepwise cleavage of sugar moieties, finally forming C-K. However, the rate of decomposition of Rb_1 seemed to differ from that of Rb_2. Therefore, Rb_1 and Rb_2 were incubated separately with rat cecal contents, and each reaction was followed by sampling at various time intervals in order to reveal more clearly the differences between decomposition modes of Rb_1 and Rb_2. The results are shown in Table II. In the case of Rb_1, the starting material (Rb_1) had already disappeared by 1 h, and decomposition had proceeded to C-K after 3 h, when Rd, the major intermediate metabolite, was no longer detectable.

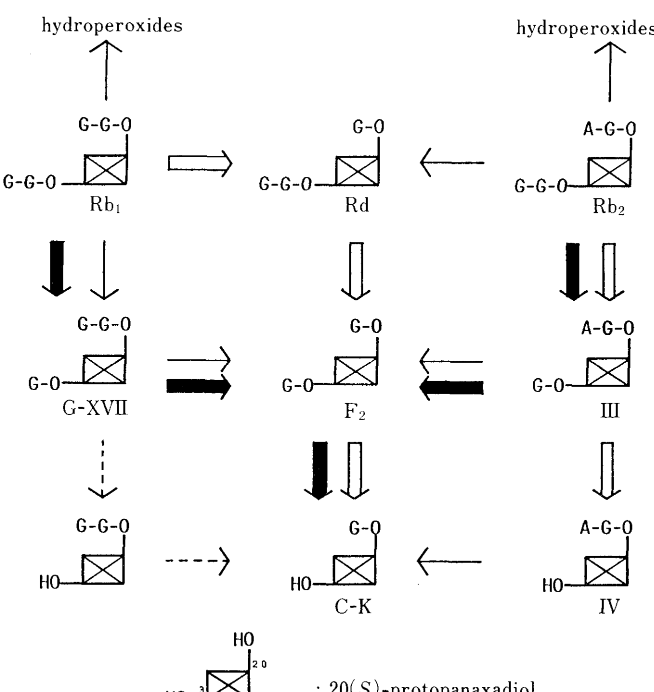

Chart 4. Decomposition Pathways of Rb_1 and Rb_2 in Rat Large Intestine or by Crude Hesperidinase

⇨, main pathway in rat large intestine; ➡, main pathway by crude hesperidinase.

On the other hand, hardly any of the Rb_2 had disappeared after 3 h, and the major intermediate metabolite (IV) still remained. Therefore, we concluded that in rat large intestine there are obvious differences in the mode of decomposition between Rb_1 and Rb_2, both belonging to the 20(*S*)-ppd saponins. These results led us to consider that because the sugar moieties of Rb_1 are all glucose and those of Rb_2 are glucose and arabinose, these might be responsible for the different rates of decomposition. Also, it was speculated that decomposition of 20(*S*)-ppd

saponins began with cleavage of the terminal sugar moiety at the C-20 hydroxyl group, followed by the terminal sugar moiety at the C-3 hydroxyl group by β-glucosidase present in the rat large intestine. However, in Rb_2, one molecule of arabinose as the terminal sugar moiety at the C-20 hydroxyl group resists attack by β-glucosidase. As a result, hydrolysis of the glycoside chain of Rb_2 probably occurs predominantly at the C-3 hydroxyl group. This assumption is strongly supported by the observation that the major intermediary metabolite of Rb_1 was Rd, despite the fact that the sugar moieties of Rb_1 are all glucose, and also that in the case of Rb_2, the disappearance of Rd was fast.

Furthermore, in order to clarify some similarities and differences between decomposition in the rat large intestine and enzymatic hydrolysis, Rb_1 and Rb_2 were decomposed by crude hesperidinase. As shown in Fig. 2, Rb_1 was decomposed to G-XVII, F_2 and C-K, and Rb_2 was decomposed to III, F_2 and C-K. Consequently, we consider that β-glucosidase present in the rat large intestine is distinct from crude hesperidinase. The patterns of decomposition of Rb_1 and Rb_2 described above are summarized in Chart 4.

Finally, we investigated the decomposition modes of Rb_1 and Rb_2 in the rat large intestine, particularly noting whether enteric enzymes or enteric bacteria are predominantly responsible. Rb_1 and Rb_2 were incubated with normal rat cecal contents suspended in 0.9% saline, a sterile filtrate, and a heated sterile filtrate, or tetracycline-treated cecal contents, respectively. The results are shown in Table III. The respective prosapogenins of Rb_1 and Rb_2, except for Rd and the hydroperoxides of Rb_1 and Rb_2, were not produced in the sterile filtrate. However, these prosapogenins were produced when tetracycline-treated cecal contents were used. In addition, Rd and hydroperoxides of Rb_1 and Rb_2 were not produced in the heated sterile filtrate. From these results, it seems that the respective prosapogenins of Rb_1 and Rb_2, except for Rd and the hydroperoxides of Rb_1 and Rb_2, are produced by tetracycline-resistant bacteria. Rd and the hydroperoxides of Rb_1 and Rb_2, however, seem to be produced by enteric enzymes. These results for the production of hydroperoxides were different from those reported previously.[2]

We therefore conclude that the decomposition of 20(*S*)-ppd saponins (Rb_1 and Rb_2) in the rat large intestine differs from that of 20(*S*)-ppt saponin (Rg_1) in rat stomach, and that the decomposition modes of Rb_1 and Rb_2, which also belong to the 20(*S*)-ppd saponins, are different because of variations in the terminal sugar moiety at the C-20 hydroxyl group. These findings may also be aplicable to other 20(*S*)-ppd and 20(*S*)-ppt saponins.

Acknowledgements The authors are grateful to the Korea Ginseng and Tabacco Research Institute and the Japan-Korea Red Ginseng Co., Ltd., for supply of pure Rb_2. Also we thank Dr. M. Uchida, of the Central Analytical Laboratory of this university, for measurement of FAB-MS spectra. This work was supported in part by a grant from the Medical Society for Red Ginseng Research.

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Studies on the Chemical Constituents of the Bulbs of *Lilium mackliniae*

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Chemical study on the bulbs of *Lilium* (*L.*) *mackliniae* has led to the isolation of a total of 27 compounds, including nine new phenolics. The structures of the new compounds have been shown by spectral analysis and chemical degradation to be 4,4′-di-*O*-acetyl-3,6′-di-*O*-feruloylsucrose, 3,4′-di-*O*-*p*-coumaroylsucrose, 3,6′-di-*O*-*p*-coumaroylsucrose, 2′-*O*-acetyl-3,4′-di-*O*-*p*-coumaroylsucrose, 3′-*O*-acetyl-3,4′-di-*O*-*p*-coumaroylsucrose, (2*S*)-1-*O*-*p*-methoxycinnamoyl-2-*O*-*β*-D-glucopyranosylglycerol (methylregaloside D), (2*S*)-1-*O*-cinnamoyl-2-*O*-*β*-D-glucopyranosylglycerol (regaloside J), (2*S*)-l-*O*-caffeoyl-2-*O*-*β*-D-glucopyranosylglycerol (regaloside K) and (2*R*)-1-*O*-*β*-D-glucopyranosyl-2-*O*-caffeoylglycerol (regaloside L). In addition, several previously reported phenolic glycosides, steroidal saponins and steroidal alkaloid have been isolated and identified. The presence of the steroidal alkaloid supported the idea that *L. mackliniae* is taxonomically related to the genus *Fritillaria*.

Keywords *Lilium mackliniae*; Liliaceae; phenolic glycoside; phenylpropanoid sucrose ester; ferulic acid, *p*-coumaric acid; phenolic glycerol glucoside; regaloside; bulb

Lilium (*L.*) *mackliniae* (Liliaceae) is native to northeastern India and Burma. It has in the past been included within the genus *Nomocharis* and is still commonly called "Manipur Nomocharis."[1] The bulbs of this plant are broadly ovoid with reddish-brown scales, and have a significantly bitter taste. No chemical analysis, however, has been done on the plant. As part of a systematic study on the constituents of the bulbs of the genus *Lilium*,[2] we have now undertaken an investigation of the fresh bulbs of *L. mackliniae* and isolated new phenolic compounds together with several known phenolics, steroidal saponins and a steroidal alkaloid. The structures of the new constituents have been established on the basis of spectroscopic analysis and chemical degradations.

Standard extraction and fractionation of the bulbs of *L. mackliniae* as shown in Chart 1 eventually gave compounds **1**—**27**.

Compounds **1**—**9** are phenylpropanoid sucrose esters, and the structures of **1**, **2**, **4** and **5** were identified as 3,6′-di-*O*-feruloylsucrose,[3] 4-*O*-acetyl-3,6′-di-*O*-feruloylsucrose,[3a] 3-*O*-feruloyl-6′-*O*-(4-*O*-*β*-D-glucopyranosylferuloyl)sucrose[4] and 4-*O*-acetyl-3-*O*-feruloyl-6′-*O*-(4-*O*-*β*-D-glucopyranosylferuloyl)sucrose,[5] respectively, by infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra, and by direct thin-layer chromatography (TLC) comparison with authentic samples.

Compound **3** was obtained as a white amorphous powder, $[\alpha]_D$ −75.2° (EtOH). The secondary ion mass spectrum (SI-MS) showed a quasimolecular ion peak at *m*/*z* 779 $[M-H]^+$. The IR spectrum showed absorption bands of hydroxyl group(s), carbonyl group(s), double bond(s) and aromatic ring(s). The ^{1}H-NMR and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were coincident with the existence of two feruloyl and two alcoholic acetyl moieties and a sucrose moiety. On alkaline methanolysis of **3** with 3% sodium methoxide in methanol, **3** liberated methyl ferulate and sucrose, confirming its constituents. The fundamental structure of **3** was diferuloylsucrose with two alcoholic acetyl groups. On comparison with the ^{1}H-NMR spectra between **3** and sucrose in pyridine-d_5, the signals assignable to the fructose H-3 and H-4, and the glucose H-4′ and H_2-6′ were shifted to a lower field by *O*-acylation. Acetylation of **3** with acetic anhydride in pyridine yielded the corresponding peracetate (**3a**). The IR, ^{1}H- and ^{13}C-NMR spectra, and the TLC behavior of **3a** fully agreed with those of the peracetate of 3,6′-di-*O*-feruloylsucrose (**1**).[3a] The structure of **3** was characterized as 4,4′-di-*O*-acetyl-3,6′-di-*O*-feruloylsucrose.

The IR, ^{1}H- and ^{13}C-NMR spectra and alkaline methanolysis proved the structural features of **6** and **7** to be di-*O*-*p*-coumaroylsucrose and those of **8** and **9** to be di-*O*-*p*-coumaroylsucrose with an acetyl group. In the ^{1}H-NMR spectra, downfield shifts from sucrose due to acylation were observed at the following signals in each compound; the fructose H-3 and the glucose H-4′ in **6**, the fructose H-3 and the glucose H_2-6′ in **7**, the fructose H-3, the glucose H-2′ and H-4′ in **8**, and the fructose H-3, the glucose H-3′ and H-4′ in **9**. The spectral data and TLC behavior of the peracetates of **8** and **9** were consistent with those of the peracetate (**6a**) of **6**. The above data identified the structures of **6**—**9** as 3,4′-di-*O*-*p*-coumaroylsucrose, 3,6′-di-*O*-*p*-coumaroylsucrose, 2′-*O*-acetyl-3,4′-di-*O*-*p*-coumaroylsucrose and 3′-*O*-acetyl-3,4′-di-*O*-*p*-coumaroylsucrose, respectively.

Compounds **10**—**20** are phenylpropanoid glycerol glucosides, and **10**—**12** and **16**—**19** were identified as regaloside B,[6] regaloside D,[7] regaloside G,[8] regaloside A,[6] regaloside C,[4] regaloside F[7] and regaloside H,[9] respectively, by their specific rotations, and by the IR and ^{1}H-NMR spectra.

The SI-MS of **13** showed a molecular ion peak at *m*/*z* 414. All the spectral data indicated that **13** is a 1-*O*-acyl-2-*O*-glucosylglycerol.[7] Acetylation of **13** by the usual manner yielded the corresponding pentaacetate (**13a**), the ^{1}H-NMR spectrum of which showed signals due to five alcoholic acetyl groups. On treatment of **13** with *β*-glucosidase, **13** was hydrolysed to yield D-glucose and 1-*O*-*p*-methoxycinnamoylglycerol (**13b**), the specific rotation of which agreed with that of (2*S*)-1-*O*-*p*-methoxycinnamoylglycerol obtained from methylregaloside A by enzymatic hydrolysis.[4] Thus, the structure of **13** was confirmed to be (2*S*)-1-*O*-*p*-methoxycinnamoyl-2-*O*-*β*-D-glucopyranosylglycerol, designated as methylregaloside D.

The spectral data of **14** and **15** was essentially analogous to that of **11**—**13**. The ^{1}H- and ^{13}C-NMR spectra revealed that the aromatic acid constituting **14** was cinnam-

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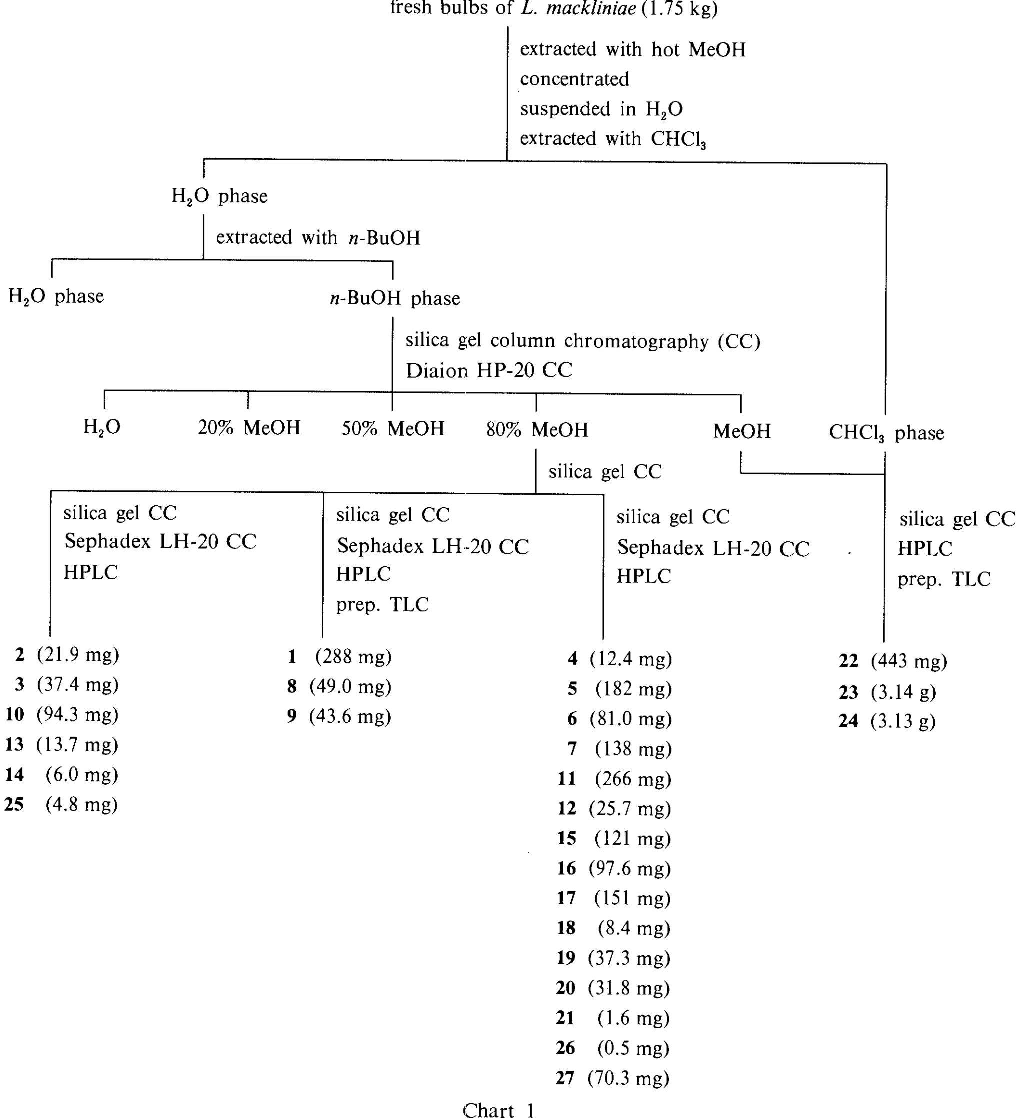

Chart 1

ic acid, and that constituting **15** was caffeic acid. On acetylation, **14** gave the corresponding pentaacetate (**14a**), and **15** gave the heptaacetate (**15a**). Enzymatic hydrolysis of **15** gave D-glucose and 1-*O*-caffeoylglycerol (**15b**), the specific rotation of which agreed with that of (2*S*)-1-*O*-caffeoylglycerol obtained from regaloside C. The structure of **15** was deduced to be (2*S*)-1-*O*-caffeoyl-2-*O*-*β*-D-glucopyranosylglycerol (regaloside K). Enzymatic hydrolysis of **14** could not be carried out because of its low yield. The structure of **14**, however, must be (2*S*)-1-*O*-cinnamoyl-2-*O*-*β*-D-glucopyranosylglycerol (regaloside J) as the ^{1}H- and ^{13}C-NMR signals arising from the glycerol moiety were in excellent agreement with those of **11**—**13** and **15**.

The ^{1}H- and ^{13}C-NMR spectral data of **20** were quite similar to those of **19**, except for the signals arising from the aromatic ring. Alkaline methanolysis showed the aromatic acid constituting **20** to be caffeic acid. On acetylation, **20** formed the corresponding heptaacetate (**20a**). In the ^{1}H-NMR spectrum of **20**, the carbinyl proton at the glycerol C-2 was deshielded by 1.07 ppm as compared with that of regaloside C[4] to appear at δ 5.13 (m), confirming the caffeic acid linkage to the glycerol C-2 hydroxyl position. The glycerol glucoside hexaacetate obtained by the alkaline methanolysis followed by acetylation of **20** was identified as lilioside C hexaacetate.[6,10] Thus, the structure of **20** was determined to be (2*R*)-1-*O*-*β*-D-glucopyranosyl-2-*O*-caffeoylglycerol (regaloside L).

Compound **21** is a minor component. The SI-MS showed a quasimolecular ion peak at *m/z* 495 [M+Na]$^{+}$. The ^{1}H-NMR spectrum indicated the presence of an allyl group, a 1,3,4-trisubstituted aromatic ring, a methoxyl group, and a disaccharide moiety. The sugar sequence, L-rhamnopyranosyl-(1→6)-D-glucopyranose was readily recognized by the ^{13}C-NMR spectrum. The above properties are in good agreement with those of eugenol 4-*O*-α-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside, which was artificially produced by the cell cultures of *Eucalyptus perriniana* with eugenol as a substrate.[11] This is the first isolation of eugenol rhamnosylglucoside from a natural plant source.

Compounds **22** and **23** are 27-hydroxyspirostanol saponins containing a 3-hydroxy-3-methylglutaroyl moiety at the C-27 hydroxyl position. The structures were assigned as (25*R*)-27-*O*-(3-hydroxy-3-methylglutaroyl)spirost-5-ene-3*β*,27-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside (brownioside)[12] and (25*R*)-27-*O*-(3-hydroxy-3-methylglutaroyl)spirost-5-ene-3*β*,27-diol 3-*O*-α-L-

	R^1	R^2
1	H	H
2	Ac	H
3	Ac	Ac

	R
4	H
5	Ac

	R^1	R^2
6	H	H
8	Ac	H
9	H	Ac

	R^1	R^2	R^3
10	OH	H	Ac
11	OH	H	H
12	OH	OMe	H
13	OMe	H	H
14	H	H	H
15	OH	OH	H

	R
16	H
17	OH
18	OMe

	R
19	H
20	OH

	R
22	H
23	Glc

Chart 2

rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside[2b,12] by the IR, ^{1}H-NMR and ^{13}C-NMR spectra, and by direct TLC comparison with authentic samples. Compound **24** is a steroidal alkaloid glycoside and the structure was assigned as solanidine 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside,[13] by IR, ^{1}H-NMR and ^{13}C-NMR spectra, and by direct TLC comparison with an authentic sample. The known compounds, **25**—**27** were identified as (±)-1-*O*-feruloylglycerol,[14] adenosine and *p*-coumaric acid 4-*O*-β-D-glucopyranoside[15] by IR and ^{1}H-NMR spectra.

Compounds **3**, **6**—**9**, **13**—**15** and **20** are new compounds. Easy acyl migration between the C-2 and C-3 hydroxyl groups, and from the C-4 to the C-6 in D-glucose occurred often.[16] Compounds **7**, and **8** or **9** may be artificial products as a result of acyl wandering during extraction and purification procedures. Our previous studies on the bulbs of the genus *Lilium* have resulted in the isolation of sucrose derivatives combined with aromatic acids as the common constituents of all the species investigated. The aromatic

TABLE I. ^{1}H-NMR Spectral Data for **3**, **3a**, **6**, **6a**, **7**, **7a**, **8**, **9** and Sucrose[a]

H	**3**	**3a**	**6**	**6a**	**7**
1a	4.35 d (12.7)	4.45—4.15	4.38 d (12.4)	4.42—4.17	4.28 d (12.3)
1b	4.25 d (12.7)		4.32 d (12.4)		4.25 d (12.3)
3	6.46 d (5.5)	5.60 d (6.1)	6.40 d (7.0)	5.63 d (6.8)	6.37 d (7.8)
4	6.21 dd (5.5, 5.5)	5.47 dd (6.1, 6.1)	5.31 dd (7.0, 7.0)	5.51 dd (6.8, 6.8)	5.30 dd (7.8, 7.8)
5	4.73—4.67	4.45—4.15	4.69 ddd (7.0, 5.6, 3.5)	4.42—4.17	4.70 ddd (7.8, 6.2, 3.5)
6a	4.53 dd (12.0, 6.2)	4.45—4.15	4.48 dd (12.2, 5.6)	4.42—4.17	4.57 dd (12.0, 6.2)
6b	4.46 dd (12.0, 5.8)		4.36 dd (12.2, 3.5)		4.47 dd (12.0, 3.5)
1′	6.06 d (3.5)	5.71 d (3.6)	6.14 d (3.7)	5.73 d (3.6)	6.12 d (3.7)
2′	4.16 dd (9.6, 3.5)	4.94 dd (9.5, 3.6)	4.18 dd (9.5, 3.7)	4.96 dd (9.6, 3.6)	4.12 dd (9.8, 3.7)
3′	4.64 dd (9.6, 9.6)	5.46 dd (9.5, 9.5)	4.78 dd (9.5, 9.5)	5.18 dd (9.6, 9.6)	4.62 dd (9.8, 9.8)
4′	5.53 dd (9.6, 9.6)	5.06 dd (9.5, 9.5)	5.76 dd (9.5, 9.5)	5.58 dd (9.6, 9.6)	4.09 dd (9.8, 9.8)
5′	[b]	4.45—4.15	4.92 ddd (9.5, 5.5, 1.8)	4.42—4.17	4.98 ddd (9.8, 6.7, 1.2)
6′a	4.83 dd (12.1, 1.8)	4.45—4.15	4.31 dd (12.2, 1.8)	4.42—4.17	5.22 dd (12.3, 1.2)
6′b	4.70 dd (12.1, 5.7)		4.14 dd (12.2, 5.5)		4.70 dd (12.3, 6.7)
Aromatic protons	7.40—7.00	7.30—7.00	7.58 d (8.6)	7.62 d (8.6)	7.58 d (8.6)
			7.44 d (8.6)	7.44 d (8.6)	7.56 d (8.6)
			7.19 d (8.6)	7.12 d (8.6)	7.07 d (8.6)
			7.08 d (8.6)	7.10 d (8.6)	7.04 d (8.6)
trans Alkene protons	8.05 d (15.8)	7.71 d (16.0)	8.07 d (15.9)	7.75 d (16.0)	8.03 d (15.9)
	8.04 d (15.8)	7.62 d (16.0)	7.87 d (15.9)	7.62 d (16.0)	7.94 d (15.9)
	6.96 d (15.8)	6.50 d (16.0)	6.67 d (15.9)	6.49 d (16.0)	6.77 d (15.9)
	6.73 d (15.8)	6.45 d (16.0)	6.36 d (15.9)	6.20 d (16.0)	6.56 d (15.9)
OMe	3.84 s	3.90 s			
	3.79 s	3.87 s			
Ac	1.96 s	2.32 s		2.29 s	
	1.86 s	2.31 s		2.26 s	
		2.12 s		2.12 s	
		2.11 s		2.11 s	
		2.09 s		2.10 s	
		2.07 s		2.07 s	
		1.97 s		2.05 s	
		1.89 s		1.93 s	

H	**7a**	**8**	**9**	Sucrose
1a	4.39—4.11	4.42 d (11.5)	4.28 d (12.4)	4.35—4.23
1b		4.21 d (11.5)	4.25 d (12.4)	
3	5.60 d (6.4)	6.53 d (7.9)	6.37 d (7.5)	4.97 d (7.9)
4	5.49 dd (6.4, 6.4)	5.37 dd (7.9, 7.9)	5.28 dd (7.5, 7.5)	5.01 dd (7.9, 7.9)
5	4.39—4.11	4.68 ddd (7.9, 5.2, 2.8)	4.64 ddd (7.5, 5.5, 3.4)	4.48
6a	4.39—4.11	4.45 dd (12.2, 5.2)	4.44 dd (12.1, 5.5)	4.35—4.23
6b		4.37 dd (12.2, 2.8)	4.35 dd (12.1, 3.4)	
1′	5.71 d (3.6)	6.31 d (3.6)	6.18 d (3.7)	6.17 d (3.8)
2′	4.94 dd (10.0, 3.6)	5.40 dd (10.0, 3.6)	4.21 dd (9.7, 3.7)	4.14 dd (9.3, 3.8)
3′	5.45 dd (10.0, 10.0)	4.90 dd (10.0, 10.0)	6.17 dd (9.7, 9.7)	4.64 dd (9.3, 9.3)
4′	5.07 dd (10.0, 10.0)	5.82 dd (10.0, 10.0)	5.78 dd (9.7, 9.7)	4.18 dd (9.3, 9.3)
5′	4.39—4.11	4.91 ddd (10.0, 6.0, 1.9)	4.94 ddd (9.7, 4.8, 2.2)	4.73
6′a	4.39—4.11	4.30 dd (12.1, 1.9)	4.28 dd (12.1, 2.2)	4.48
6′b		4.14 dd (12.1, 6.0)	4.10 dd (12.1, 4.8)	4.35—4.23
Aromatic protons	7.63 d (8.8)	7.62 d (8.6)	7.58 d (8.6)	
	7.56 d (8.8)	7.45 d (8.6)	7.45 d (8.6)	
	7.13 d (8.8)	7.18 d (8.6)	7.14 d (8.6)	
	7.11 d (8.8)	7.11 d (8.6)	7.08 d (8.6)	
trans Alkene protons	7.76 d (16.0)	8.06 d (15.9)	8.11 d (15.9)	
	7.66 d (16.0)	7.85 d (15.9)	7.90 d (15.9)	
	6.50 d (16.0)	6.77 d (15.9)	6.75 d (15.9)	
	6.46 d (16.0)	6.36 d (15.9)	6.42 d (15.9)	
OMe				
Ac	2.30 s	1.98 s	1.83 s	
	2.29 s			
	2.12 s			
	2.10 s			
	2.08 s			
	2.07 s			
	1.97 s			
	1.88 s			

a) Spectra of **3**, **6**—**9** and sucrose were measured in C_5D_5N, and those of **3a**, **6a** and **7a** in $CDCl_3$. *b*) Signal is unclear due to overlapping with H_2O signal. *J* values in parentheses are expressed in Hz. All the assignments were confirmed by double resonance experiments.

TABLE II. ^{13}H-NMR Spectral Data for **3**, **6**—**9** and Sucrose[a)]

C	3	6	7	8	9	Sucrose[b)]
1	65.0	65.7	65.2[c)]	65.1	65.2	64.6
2	106.4	105.3	104.8	105.3	105.3	105.4
3	77.4	80.1	79.8	79.4	79.8	79.8
4	77.4	74.0	74.0	74.4	74.8	75.5
5	83.7	85.2	84.7	84.7	84.9	84.0
6	63.2	62.6[c)]	63.3	62.6[c)]	62.6[c)]	62.8[c)]
1′	93.3	93.0	93.0	90.3	92.8	93.3
2′	73.0	73.3	73.2	73.4	70.1	73.1
3′	72.5[c)]	72.7[d)]	75.1	69.7	73.7	74.8[d)]
4′	72.3[c)]	73.0[d)]	71.7	72.8[d)]	70.9	71.6
5′	70.1	72.9[d)]	72.3	72.6[d)]	72.5	74.6[d)]
6′	63.9	62.3[c)]	65.0[c)]	62.2[c)]	61.6[c)]	62.3[c)]
1″, 1‴	126.6	126.2, 126.1	126.3, 126.2	126.1, 125.9	126.4, 125.9	
2″, 2‴	111.7, 111.6	130,8, 130.7	130.9, 130.7	130.9, 130.8	131.0	
3″, 3‴	149.0[d)]	116.8	116.7	116.9	116.8, 116.7	
4″, 4‴	151.2,[d)] 151.1[d)]	161.5	161.4, 161.3	161.7, 161.6	161.6, 161.4	
5″, 5‴	116.8	116.8	116.7	116.9	116.8, 116.7	
6″, 6‴	124.2, 124.0	130.8, 130.7	130.9, 130.7	130.9, 130.8	131.0	
7″, 7‴	146.9, 146.2	145.9, 145.6	145.9, 145.3	146.1, 145.8	146.3, 146.1	
8″, 8‴	115.2, 114.5	115.1, 115.0	115.3, 114.8	114.7, 114.6	115.0, 114.2	
9″, 9‴	167.6, 166.2	167.3, 166.8	167.7, 167.0	167.2, 167.1	167.0, 166.8	
OMe	56.1 55.9					
Ac	170.4, 20.8 170.3, 20.7			170.9, 20.9	170.4, 20.8	

a) Spectra were measured in C_5D_5N. *b*) Data was quoted from literature, *Chem. Pharm. Bull.*, **34**, 5005 (1986). *c*, *d*) Assignments with the same superscripts may be reversed in each vertical column.

TABLE III. ^{1}H-NMR Spectral Data for **13**—**15** and **20**[a)]

	13	14	15	20
1a	4.37 dd (11.6, 4.8)	4.39 dd (11.7, 4.8)	4.36 dd (11.6, 4.8)	4.04 dd (10.9, 5.5)
1b	4.31 dd (11.6, 6.2)	4.33 dd (11.7, 6.0)	4.30 dd (11.6, 6.3)	3.83 dd (10.9, 5.4)
2	4.07 m	4.06 m	4.05 m	5.13 m
3a	3.73 d (5.0)	3.73 d (5.0)	3.72 d (5.0)	3.81 dd (11.8, 4.7)
3b				3.77 dd (11.8, 5.5)
Aromatic protons	7.56 d (8.8) 6.95 d (8.8)	7.39—7.63	7.05 d (1.8) 6.96 dd (8.2, 1.8) 6.78 d (8.2)	7.05 d (2.0) 6.95 dd (8.1, 2.0) 6.78 d (8.1)
trans Alkene protons	7.69 d (16.0) 6.40 d (16.0)	7.74 d (16.1) 6.56 d (16.1)	7.59 d (15.9) 6.28 d (15.9)	7.60 d 815.9) 6.29 d (15.9)
1″	4.49 d (7.8)	4.49 d (7.8)	4.48 d (7.8)	4.32 d (7.7)
2″	3.23 dd (9.0, 7.8)	3.23 dd (9.1, 7.8)	3.23 dd (9.0, 7.8)	3.19 dd (9.0, 7.8)
3″	3.42—3.27	3.40—3.27	3.40—3.26	3.37—3.28
4″	3.42—3.27	3.40—3.27	3.40—3.26	3.37—3.28
5″	3.42—3.27	3.40—3.27	3.40—3.26	3.37—3.28
6″a	3.87 dd (11.9, 1.6)	3.87 dd (11.9, 1.6)	3.87 br d (11.8)	3.86 dd (12.1, 1.6)
6″b	3.67	3.67	3.67	3.67 dd (12.1, 5.1)
OMe	3.83 s			

a) Spectra were measured in CD_3OD. *J* values in parentheses are expressed in Hz.

acid esters of the sucrose derivatives were usually limited only to ferulic acid linked to the fructose C-3 and the glucose C-6′ hydroxyl positions. Compounds **6**, **8** and **9** are distinctive in carring *p*-coumaric acids at the fructose C-3 and the glucose C-4′ hydroxyl positions. S. G. How proposed a classification of the genus *Lilium* into a total of 13 sections,[17)] in which *L. mackliniae* was divided into the Lophophorum section. The section is closely related to the genera *Fritillaria* and *Nomocharis*. It is interesting from the view point of chemotaxonomy that *L. mackliniae* contains both the phenolic glycosides and solanidine glycoside; the former is widely distributed in the genus *Lilium* and the latter was recently detected in *Fritillaria thunbergii*[13a)] and *F. camtschatcensis*.[13b)]

Experimental

Optical rotations were measured with a JASCO DIP-360 automatic polarimeter. IR spectra were recorded on a Hitachi 260-30 or a Perkin-Elmer 1710 FTIR spectrometer, ultraviolet (UV) spectra on a Hitachi 557 spectrometer, and mass spectra (MS) on a Hitachi M-80 machine. Nuclear magnetic resonance (NMR) spectra were taken with a Bruker AM-400 spectrometer. Chemical shifts are reported in ppm (δ scale) with tetramethylsilane as an internal standard, and the following abbreviations are used: s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; br, broad. Column chromatographies were carried out on Fuji Davison Silica gel BW-300 (200—400 mesh, Fuji Davison Co., Ltd.),

TABLE IV. ^{13}C-NMR Spectral Data for 13—15 and 20[a)]

C	13	14	15	20
1	64.8	65.0	64.8	71.3
2	79.7	79.5	79.5	74.6
3	63.4	63.4	63.4	65.4
1′	128.4	137.4	127.8	127.8
2′	131.1	129.3	114.9	115.2
3′	115.5	130.1	146.9	146.9
4′	163.3	131.6	149.7	149.7
5′	115.5	130.1	116.6	116.9
6′	131.1	129.3	123.1	123.0
7′	146.6	146.7	147.4	147.4
8′	116.0	118.7	115.3	116.6
9′	168.9	168.5	169.2	168.9
1″	104.2	104.3	104.2	104.8
2″	75.1	75.1	75.1	75.1
3″	78.0	78.0	78.0	78.1
4″	71.6	71.6	71.6	71.7
5″	78.0	78.0	78.0	78.1
6″	62.7	62.7	62.7	62.8
OMe	55.9			

a) Spectra were measured in CD_3OD.

Sephadex LH-20 (25—100 μm, Pharmacia Co., Ltd.) and Diaion HP-20 (Mitsubishi-kasei Co., Ltd.). TLC was carried out on precoated Kieselgel 60 F_{254} plates (0.25 mm thick, Merck) and preparative TLC on precoated Kieselgel 60 F_{254} plates (0.5 mm thick, Merck). Spots were visualized under UV light (254 nm) irradiation and by spraying with a 10% H_2SO_4 solution, followed by heating. Alkaloid was located using the Dragendorff reagent. High performance liquid chromatography (HPLC) was performed with a CIG column system (Kusano Kagakukikai Co., Ltd.: pump, Kusano micropump KP-6S; detector, Kusano UVILOG-III) or Tosoh HPLC System (Tosoh Co., Ltd.: pump, Tosoh CCPM; detector, Tosoh RI-8010; controller, CCP controller PX-8010) equipped with a CIG pre-packed column (Kusano Kagakukikai Co., Ltd., 20 i.d. × 100 mm, octadecyl silica (ODS), 20 μm) or a Kaseisorb LC ODS-120-5 column (Tokyo Kasei Kogyo Co., Ltd., 20 i.d. × 250 mm, ODS, 5 μm).

Extraction and Isolation The bulbs of *L. mackliniae* were purchased from Heiwaen Co., Japan. Extraction and isolation procedures are shown in Chart 1. The solvent systems used were as follows: silica gel column chromatography, CH_2Cl_2–MeOH, $CHCl_3$–MeOH, $CHCl_3$–MeOH–H_2O, EtOAc–Me_2CO, EtOAc–MeOH and $CHCl_3$–MeOH–NH_3; Sephadex LH-20, MeOH; HPLC, MeOH–H_2O, MeCN–H_2O and 2-methoxyethanol–MeOH–H_2O; preparative TLC, EtOAc–MeCOEt–MeOH–H_2O (10 : 10 : 1 : 1) and $CHCl_3$–*n*-BuOH–MeOH–H_2O–AcOH (20 : 10 : 10 : 4 : 3).

4,4′-Di-*O*-Acetyl-3,6-di-*O*-feruloylsucrose (3) A white amorphous powder, $[\alpha]_D^{26}$ −75.2° (c=0.50, EtOH). SI-MS *m/z*: 779 $[M-H]^+$, 737 $[M-Ac-H]^+$. UV λ_{max}^{EtOH} nm (log ε): 235 (4.33), 300 sh (4.43), 328 (4.58). UV $\lambda_{max}^{EtOH+NaOMe}$ nm: 386. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2920 (CH), 1710 (C=O), 1620 (CH=CH), 1600, 1510 (aromatic rings), 1460, 1450, 1420, 1370, 1250, 1150, 1020, 840, 820.

Alkaline Methanolysis of 3 Methanolysis of **3** (3.0 mg) was carried out with 3% NaOMe in methanol at ambient temperature for 30 min. The reaction mexture was passed through an Amberlite IR-120B column. Methyl ferulate and sucrose were detected in the mixture by TLC. Methyl ferulate: *Rf* 0.30 (*n*-hexane–Me_2CO, 2 : 1); *Rf* 0.85 ($CHCl_3$–MeOH, 19 : 1). Sucrose: *Rf* 0.09 ($CHCl_3$–MeOH–H_2O, 20 : 10 : 1); *Rf* 0.36 (*n*-BuOH–Me_2CO–H_2O, 4 : 5 : 1).

Acetylation of 3 Compound **3** (6.1 mg) was treated with Ac_2O in pyridine at room temperature overnight and the crude acetate was subjected to a silica gel column using $CHCl_3$–EtOAc (5 : 1) to yield the peracetate (**3a**) (4.9 mg). The spectral data and TLC behavior were identical with those of 3,6-di-*O*-feruloylsucrose (**1**) octaacetate. TLC; *Rf* 0.25 ($CHCl_3$–EtOAc, 3 : 1).

3,4′-Di-*O*-*p*-coumaroylsucrose (6) A white amorphous powder, $[\alpha]_D^{28}$ −88.0° (c=0.10, MeOH). SI-MS *m/z*: 657 $[M+Na]^+$, 635 $[M+H]^+$. UV λ_{max}^{MeOH} nm (log ε): 229 (4.52), 305 sh (4.76), 313 (4.79). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 362. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2965, 2925, 2855 (CH), 1700 (C=O), 1625 (CH=CH), 1600, 1585, 1510 (aromatic rings), 1440, 1325, 1260, 1200, 1170, 1040, 1000, 855, 815, 800.

Alkaline Methanolysis of 6 Compound **6** (1.2 mg) was subjected to alkaline methanolysis as in the case of **3**. Methyl *p*-coumarate and sucrose were detected in the reaction mixture by TLC. Methyl *p*-coumarate; *Rf* 0.52 ($CHCl_3$–MeOH, 15 : 1).

Acetylation of 6 Compound **6** (4.7 mg) was acetylated with Ac_2CO in pyridine and the crude acetate was chromatographed on silica gel with *n*-hexane–Me_2CO (5 : 1) to the corresponding octaacetate (**6a**) (5.4 mg). CI (Chemical ionization)-MS *m/z* (%): 971 $[M+H]^+$ (4), 982 $[M-Ac]^+$ (100), 782 [M−acetyl-*p*-coumaroyl]$^+$ (2), 610 (8). IR ν_{max}^{KBr} cm^{-1}: 2960 (CH), 1745 (C=O), 1630 (CH=CH), 1600, 1580, 1505 (aromatic rings), 1430, 1415, 1365, 1320, 1220, 1160, 1025, 905, 835.

3,6′-Di-*O*-*p*-coumaroylsucrose (7) A white amorphous powder, $[\alpha]_D^{28}$ −72.0° (c=0.30, MeOH). SI-MS *m/z*: 657 $[M+Na]^+$, 633 $[M-H]^+$. UV λ_{max}^{MeOH} nm (log ε): 229 (4.28), 305 sh (4.51), 313 (4.57). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 362. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2920, 2850 (CH), 1690 (C=O), 1625 (CH=CH), 1600, 1580, 1510 (aromatic rings), 1445, 1370, 1325, 1255, 1200, 1160, 1040, 855, 815, 800.

Acetylation of 7 Compound **7** (3.0 mg) was acetylated with Ac_2CO in pyridine, and the crude acetate was chromatographed on silica gel with *n*-hexane–Me_2CO (3 : 1) to the corresponding octaacetate (**7a**) (2.8 mg). CI-MS *m/z* (%): 971 $[M+H]^+$ (2), 930 (10), 824 (6), 812 (7), 782 (100), 740 (10), 618 (27). IR ν_{max}^{KBr} cm^{-1}: 2960 (CH), 1750 (C=O), 1630 (CH=CH), 1600, 1580, 1505 (aromatic rings), 1430, 1415, 1365, 1315, 1225, 1165, 1035, 965, 905, 835.

2′-*O*-Acetyl-3,4′-di-*O*-*p*-coumaroylsucrose (8) A white amorphous powder, $[\alpha]_D^{26}$ −59.3° (c=0.30, MeOH). SI-MS *m/z*: 699 $[M+Na]^+$, 677 $[M+H]^+$, 659 $[M-OH]^+$, 635 $[M-Ac+H]^+$. UV λ_{max}^{MeOH} nm (log ε): 229 (4.40), 304 sh (4.62), 313 (4.67). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 362. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2950 (CH), 1700 (C=O), 1630 (CH=CH), 1605, 1585, 1515 (aromatic rings), 1445, 1375, 1330, 1265, 1205, 1170, 1050, 1010, 980, 935, 860, 830.

3′-*O*-Acetyl-3,4′-di-*O*-*p*-coumaroylsucrose (9) A white amorphous powder, $[\alpha]_D^{26}$ −5.0° (c=0.20, MeOH). SI-MS *m/z*: 675 $[M-H]^+$, 659 $[M-OH]^+$, 645, 635 $[M-Ac+H]^+$. UV λ_{max}^{MeOH} nm (log ε): 229 (4.35), 304 sh (4.59), 313 (4.64). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 362. IR ν_{max}^{KBr} cm^{-1}: 3535, 3420, 3300 (OH), 2970, 2940 (CH), 1725, 1710 (C=O), 1630 (CH=CH), 1605, 1585, 1510 (aromatic rings), 1445, 1380, 1370, 1320, 1300, 1290, 1275, 1255, 1200, 1160, 1135, 1100, 1060, 1050, 1040, 1020, 980, 950, 940, 860, 830.

Acetylation of 8 and 9 Compound **8** (20.7 mg) and **9** (22.1 mg) were acetylated with Ac_2CO in pyridine, and the crude acetates were chromatographed on silica gel with *n*-hexane–Me_2CO (3 : 1) to the corresponding peracetates (10.6 and 25.5 mg). The spectral data and TLC behaviors were identical with those of **6a**. TLC: *Rf* 0.47 (*n*-hexane–Me_2CO, 1 : 1).

(2*S*)-1-*O*-*p*-Methoxycinnamoyl-2-*O*-β-D-glucopyranosylglycerol (Methylregaloside D) (13) A white amorphous powder, $[\alpha]_D^{26}$ −19.2° (c=0.50, MeOH). SI-MS *m/z*: 414 $[M]^+$, 252 $[M-Glc]^+$, 178, 163, 161, 145, 121. UV λ_{max}^{MeOH} nm (log ε): 226 (4.12), 301 sh (4.38), 313 (4.41). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 2925 (CH), 1700 (C=O), 1620 (CH=CH), 1600, 1580, 1510 (aromatic ring), 1430, 1415, 1320, 1250, 1170, 1155, 1065, 1020, 820.

Acetylation of 13 Compound **13** (3.0 mg) was acetylated with Ac_2CO in pyridine and the crude acetates were chromatographed on silica gel with $CHCl_3$–EtOAc (5 : 1) to the corresponding peracetate (**13a**) (3.2 mg). EI (electron impact)-MS *m/z* (%): 582 $[M-Ac]^+$ (1.7), 322 (4), 277 (35), 235 (9), 205 (11), 178 (18), 161 (100), 103 (86). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2945 (CH), 1750 (C=O), 1625 (CH=CH), 1600, 1570, 1505 (aromatic ring), 1450, 1435, 1420, 1365, 1300, 1285, 1230, 1165, 1035, 980, 905, 820. ^{1}H-NMR ($CDCl_3$) δ: 7.68 (1H, d, J=16.0 Hz, H-7′), 7.50 (2H, d, J=8.6 Hz, H-2′, -6′), 6.92 (1H, d, J=8.6 Hz, H-3′, 5′), 6.30 (1H, d, J=16.0 Hz, H-8′), 5.22 (1H, dd, J=9.5, 9.5 Hz, H-3″), 5.08 (1H, dd, J=9.5, 9.5 Hz, H-4″), 5.01 (1H, dd, J=9.5, 8.1 Hz, H-2″), 4.70 (1H, d, J=8.1 Hz, H-1″), 4.33—4.12 (7H, H-1, -2, -3, -6″), 3.85 (3H, s, OMe), 3.72 (1H, m, H-5″), 2.09, 2.07, 2.02, 2.00, 1.99 (each 3H, s, Ac).

Enzymatic Hydrolysis of 13 Compound **13** (10.0 mg) was dissolved in an AcOH–AcONa buffer (pH 5) with β-glucosidase (8.0 mg), and the reaction mixture was incubated at 37 °C for 4 h. The crude products were chromatographed on silica gel with $CHCl_3$–MeOH (19 : 1→4 : 1) to yield (2*S*)-1-*O*-*p*-methoxycinnamoylglycerol (**13b**) (2.7 mg) and D-glucose (4.1 mg). Compound **13b**: a colorless syrup, $[\alpha]_D^{28}$ +8.1° (c=0.27, MeOH). EI-MS *m/z* (%): 252.0969 [M$^+$, Calcd for $C_{13}H_{16}O_5$: 252.0998] (17), 178 (34), 161 (100), 133 (27), 118 (7), 103 (8). IR ν_{max}^{KBr} cm^{-1}: 3418 (OH), 2936 (CH), 1708 (C=O), 1633 (CH=CH), 1604, 1575, 1514 (aromatic ring), 1459, 1423, 1305, 1290, 1255, 1171, 1117, 1030, 984, 829. ^{1}H-NMR (CD_3OD) δ: 7.68 (1H, d, J=16.0 Hz, H-7′), 7.55 (2H, d, J=8.7 Hz, H-2′, -6′), 6.96 (2H, d, J=8.7 Hz, H-3′, -5′), 6.41 (1H, d, J=16.0 Hz, H-8′),

4.27 (1H, dd, $J=11.4$, 4.3 Hz, H-1a), 4.17 (1H, dd, $J=11.4$, 6.3 Hz, H-1b), 3.89 (1H, m, H-2), 3.83 (3H, s, OMe), 3.62 (1H, dd, $J=11.3$, 5.4 Hz, H-3a), 3.57 (1H, dd, $J=11.3$, 5.7 Hz, H-3b). The above data completely agreed with that of the sample obtained from methylregaloside A by the same method. D-Glucose: $[\alpha]_D^{28}$ +56.1° (c=0.08, H_2O); TLC, Rf 0.15 ($CHCl_3$–MeOH–H_2O, 20:10:1), Rf 0.67 (MeOH–Me_2CO–H_2O, 4:5:1).

(2*S*)-1-*O*-Cinnamoyl-2-*O*-β-D-glucopyranosylglycerol (Regaloside J) (14) A colorless viscous syrup, $[\alpha]_D^{27}$ −19.3° (c=0.55, MeOH). CI-MS m/z (%): 292 (3), 255 (5), 223 (7.7), 2.5 (3.3), 163 (79), 145 (66), 131 (34), 127 (19), 103 (71), 93 (100). UV λ_{max}^{MeOH} nm (log ε): 217 (4.23), 223 (4.17), 276 (4.45). IR ν_{max}^{KBr} cm^{-1}: 3423 (OH), 2926 (CH), 1707 (C=O), 1637 (CH=CH), 1605, 1580, 1510 (aromatic ring), 1451, 1313, 1171, 1076, 1040, 769.

Alkaline Methanolysis of 14 Compound **14** (2.5 mg) was subjected to alkaline methanolysis as in the case of **3**. Methyl cinnamate and 2-*O*-β-D-glucopyranosylglycerol were detected in the reaction mixture by TLC. Methyl cinnamate: Rf 0.76 (n-hexane–Me_2CO, 2:1), Rf 0.38 (n-hexane–$CHCl_3$, 2:1). 2-*O*-β-D-Glucopyranosylglycerol: Rf 0.55 (MeOH–Me_2CO–H_2O, 8:12:1), Rf 0.23 ($CHCl_3$–MeOH–H_2O, 20:10:1).

Acetylation of 14 Compound **14** (3.0 mg) was acetylated with Ac_2CO in pyridine and the crude acetates were chromatographed on silica gel with $CHCl_3$–EtOAc (5:1) to the corresponding peracetate (**14a**) (4.0 mg). EI-MS m/z (%): 594 $[M]^+$ (0.3), 534 (0.3), 432 (0.3), 331 (1.7), 293 (2.5), 247 (47), 205, (6), 159 (9), 131 (100), 103 (25). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2980, 2940 (CH), 1745 (C=O), 1630 (CH=CH), 1595, 1485 (aromatic ring), 1440, 1365, 1300, 1225, 1155, 1055, 1025, 970, 900, 850, 830, 820. ^{1}H-NMR ($CDCl_3$) δ: 7.73 (1H, d, $J=16.0$ Hz, H-7′), 7.56—7.38 (5H, H-2′, -3′, -4′, -5′, -6′), 6.44 (1H, d, $J=16.0$ Hz, H-8′), 5.22 (1H, dd, $J=9.5$, 9.5 Hz, H-3″), 5.08 (1H, dd, $J=9.5$, 9.5 Hz, H-4″), 5.01 (1H, dd, $J=9.5$, 8.1 Hz, H-2″), 4.70 (1H, d, $J=8.1$ Hz, H-1″), 4.37—4.10 (7H, H-1, -2, -3, -6″), 3.72 (1H, m, H-5″), 2.09, 2.08, 2.02, 2.00, 1.99 (each 3H, s, Ac).

(2*S*)-1-*O*-Caffeoyl-2-*O*-β-D-glucopyranosylglycerol (Regaloside K) (15) A pale-yellow amorphous powder, $[\alpha]_D^{28}$ −27.4° (c=0.23, MeOH). SI-MS m/z: 461 $[M+2Na-H]^+$, 439 $[M+Na]^+$, 417 $[M+H]^+$, 369. UV λ_{max}^{MeOH} nm (log ε): 240 sh (4.06), 300 sh (4.20), 330 (4.33). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 374. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2920 (CH), 1700 (C=O), 1630 (CH=CH), 1600, 1520 (aromatic ring), 1420, 1380, 1280, 1180, 1160, 1110, 1070, 1030, 980, 850, 810, 700.

Acetylation of 15 Compound **15** (5.7 mg) was acetylated with Ac_2CO in pyridine and the crude acetates were chromatographed on silica gel with n-hexane–Me_2CO (2:1) to the corresponding peracetate (**15a**) (6.9 mg). EI-MS m/z (%): 668 $[M-Ac]^+$ (1.5), 623 (2.5), 543 (1.5), 503 (3.5), 429 (8), 363 (12), 331 (32), 281 (15), 247 (7), 205 (20), 169 (90), 119 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3020, 2945 (CH), 1750 (C=O), 1630 (CH=CH), 1600, 1495 (aromatic ring), 1420, 1365, 1225, 1170, 1060, 1030, 1005, 980, 900, 820. ^{1}H-NMR ($CDCl_3$) δ: 7.65 (1H, d, $J=15.9$ Hz, H-7′), 7.43 (1H, dd, $J=8.4$, 2.0 Hz, H-6′), 7.39 (1H, d, $J=2.0$ Hz, H-2′), 7.25 (1H, d, $J=8.4$ Hz, H-5′), 6.38 (1H, d, $J=15.9$ Hz, H-8′), 5.22 (1H, dd, $J=9.5$, 9.5 Hz, H-3″), 5.08 (1H, dd, $J=9.5$, 9.5 Hz, H-4″), 5.01 (1H, dd, $J=9.5$, 8.0 Hz, H-2″), 4.70 (1H, d, $J=8.0$ Hz, H-1″), 4.36—4.12 (7H, H-1, -2, -3, -6″), 3.72 (1H, m, H-5″), 2.32, 2.31, 2.09, 2.08, 2.02, 2.00, 1.99 (each 3H, s, Ac).

Enzymatic Hydrolysis of 15 Compound **15** (11.7 mg) was dissolved in an AcOH–AcONa buffer (pH 5) with β-glucosidase (8.0 mg), and the reaction mixture was incubated at 37 °C for 2.5 h. The crude products were chromatographed on silica gel with $CHCl_3$–MeOH (19:1→4:1) to yield (2*S*)-1-*O*-caffeoylglycerol (**15b**) (1.6 mg) and D-glucose (4.0 mg). Compound **15b**: a pale-yellow syrup, $[\alpha]_D^{28}$ +9.1° (c=0.02, MeOH). EI-MS m/z (%): 254 $[M]^+$ (17), 180 (23), 163 (91), 145 (6), 134 (38), 117 (10), 89 (22), 61 (100). IR ν_{max}^{KBr} cm^{-1}: 3520, 3360 (OH), 2975, 2890 (CH), 1685 (C=O), 1635 (CH=CH), 1610, 1595, 1535 (aromatic ring), 1450, 1370, 1300, 1290, 1250, 1185, 1115, 1055, 1015, 970, 930, 875, 840, 805. ^{1}H-NMR (CD_3OD) δ: 7.59 (1H, d, $J=15.9$ Hz, H-7′), 7.04 (1H, d, $J=2.1$ Hz, H-2′), 6.95 (1H, d, $J=8.2$, 2.1 Hz, H-6′), 6.78 (1H, d, $J=8.2$ Hz, H-5′), 6.29 (1H, d, $J=15.9$ Hz, H-8′), 4.26 (1H, dd, $J=11.4$, 4.4 Hz, H-1a), 4.16 (1H, dd, $J=11.4$, 6.3 Hz, H-1b), 3.89 (1H, m, H-2), 3.62 (1H, dd, $J=11.3$, 5.4 Hz, H-3a), 3.59 (1H, dd, $J=11.3$, 5.9 Hz, H-3b). Above data completely agreed with those of the sample obtained from regaloside C by the same method.

(2*S*)-1-*O*-β-D-Glucopyranosyl-2-*O*-caffeoylglycerol (Regaloside L) (20) A pale-yellow amorphous powder, $[\alpha]_D^{28}$ −29.1° (c=0.55, MeOH). CI-MS m/z (%): 417 $[M+H]^+$ (17), 369 (20), 242 (22), 208 (52), 179 (48), 163 (57), 147 (59), 129 (100), 111 (96). UV λ_{max}^{MeOH} nm (log ε): 243 sh (3.74), 302 sh (3.94), 325 (4.02). UV $\lambda_{max}^{MeOH+NaOMe}$ nm: 372. IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 2963, 2927 (CH), 1701 (C=O), 1631 (CH=CH), 1605, 1517 (aromatic ring), 1450, 1377, 1262, 1165, 1078, 1034, 857, 805.

Acetylation of 20 Compound **20** (3.0 mg) was acetylated with Ac_2CO in pyridine and the crude acetates were chromatographed on silica gel with n-hexane–Me_2CO (2:1) to the corresponding peracetates (3.6 mg). EI-MS m/z (%): 711 $[M+H]^+$ (0.4), 668 $[M-Ac]^+$ (1.4), 624 (4), 477 (2.5), 434 (1.2), 385 (1.5), 331 (47), 278 (12), 211 (9), 169 (100), 109 (53). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3030, 2935 (CH), 1750 (C=O), 1630 (CH=CH), 1600, 1580, 1495 (aromatic ring), 1420, 1365, 1225, 1170, 1105, 1030, 980, 900, 825. ^{1}H-NMR ($CDCl_3$) δ: 7.65 (1H, d, $J=16.0$ Hz, H-7′), 7.42 (1H, dd, $J=8.4$, 2.1 Hz, H-6′), 7.38 (1H, d, $J=2.1$ Hz, H-2′), 7.24 (1H, d, $J=8.4$ Hz, H-5′), 6.37 (1H, d, $J=16.0$ Hz, H-8′), 5.31 (1H, m, H-2), 5.21 (1H, dd, $J=9.7$, 9.7 Hz, H-3″), 5.08 (1H, dd, $J=9.7$, 9.7 Hz, H-4″), 5.00 (1H, dd, $J=9.7$, 8.0 Hz, H-2″), 4.57 (1H, d, $J=8.0$ Hz, H-1″), 4.37 (1H, dd, $J=12.0$, 3.8 Hz, H-3a), 4.25 (1H, dd, $J=12.1$, 4.5 Hz, H-6″a), 4.24 (1H, dd, $J=12.0$, 6.1 Hz, H-3b), 4.14 (1H, dd, $J=12.1$, 2.3 Hz, H-6″b), 4.02 (1H, dd, $J=11.2$, 4.6 Hz, H-1a), 3.79 (1H, dd, $J=11.2$, 5.3 Hz, H-1b), 3.70 (1H, m, H-5″), 2.31, 2.30, 2.09, 2.07, 2.04, 2.02, 2.00 (each 3H, s, Ac).

Alkaline Methanolysis Followed by Acetylation of 20 Compound **20** (4.0 mg) was hydrolysed with 3% NaOMe in MeOH at room temperature for 1.5 h. The crude hydrolysate was passed through an Amberlite IR-120B column. Methyl caffeate was detected in the reaction mixture by TLC; Rf 0.19 ($CHCl_3$–MeOH, 15:1). The mixture was acetylated with Ac_2CO in pyridine and the crude product was subjected to preparative HPLC using MeOH–H_2O (19:1) to yield glycerol glucoside hexaacetate, which was identified as lilioside C hexaacetate.

Eugenol 4-*O*-α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside (21) A pale-yellow amorphous powder, $[\alpha]_D^{28}$ −72.7° (c=0.11, MeOH). SI-MS m/z: 495 $[M+Na]^+$. UV λ_{max}^{MeOH} nm (log ε): 280 (3.82).

Acknowledgments The authors are grateful to Dr. Y. Shida and Mrs. Y. Katou for measurement of the mass spectra. Thanks are due to Dr. T. Furuya for providing the authentic sample, eugenol 4-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

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High-Performance Liquid Chromatographic Determination of Peptides Released by Tryptic Degradation from Opioid Peptide Precursors in Rat Brain

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A high-performance liquid chromatographic method involving postcolumn fluorescence derivatization is described for the quantification of five fragment peptides (methionine–enkephalin–Thr–Ser–Glu–Lys, methionine–enkephalin–Lys, methionine–enkephalin–Arg, leucine–enkephalin–Lys and leucine–enkephalin–Arg) released by tryptic digestion from the opioid peptide precursors (proopiomeranocortin and proenkephalins A and B) in rat brain tissues. The tissue proteins containing the precursors are hydrolyzed with trypsin to the fragment peptides. The peptides are separated on an Asahipak ODP-50 column and on-line detected fluorometrically by using hydroxylamine, cobalt(II) and borate buffer reagents. The detection limits (S/N = 3) for the peptides are 0.7—2.8 pmol per 100 μl injected. The distribution of the precursors in the brain tissues was also discussed on the basis of the determined values of the fragment peptides.

Keywords opioid peptide precursor; brain tissue; tryptic degradation; fragment peptide; postcolumn fluorescence derivatization; high-performance liquid chromatography

It has been recognized that naturally occurring opioid peptides involving enkephalins, methionine–enkephalin (ME, Tyr–Gly–Gly–Phe–Met) and leucine–enkephalin (LE, Tyr–Gly–Gly–Phe–Leu), are derived from three different precursors, namely proopiomeranocortin, and proenkephalins A and B (Table I).[1-4] The opioid peptide moieties of these precursor proteins are typically flanked on both sides by basic amino acid residues which appear to be a processing signal for the trypsin-like enzyme.[5]

Clearly, to arrive at an understanding of the details of the biosynthesis of opioid peptides in the brain, sensitive and convenient methods for estimating the distribution of the opioid peptide precursors in the brain are necessary. Radioimmunoassay (RIA) has usually been applied for the assay of the opioid peptide precursors. The production of antiserum directed against a purified bovine ME precursor (probably corresponding to proenkephalin A) was reported.[6] However, the antibody cross-reacted with other proteins having no enkephalin sequence. In the other RIA methods, a fragment peptide, ME–Arg released from proenkephalin A by tryptic hydrolysis was determined for the estimation of the precursor in rat brain.[7,8] However, no methods based on RIA are available to estimate the amounts of proopiomeranocortin and proenkephalin B. Recently, a method based on high-performance liquid chromatography (HPLC) with off-line mass spectrometric detection was utilized for the determination of ME and LE yielded enzymatically from the precursor proteins in human cerebrospinal fluid, where two enzymes, trypsin and carboxypeptidase B, were used for the liberation of ME and LE.[9]

TABLE I. Partial Amino Acid Sequences of the Opioid Peptide Precursors and the Fragment Peptides Released by Tryptic Digestion (Underlined)

Proopiomeranocortin
- Lys–Arg^{103}–Tyr–Gly–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser

Proenkephalin A
- –Lys–Arg^{99}–Tyr–Gly–Gly–Phe–Met–Lys–Arg–Tyr–Gly–Gly–Phe–Met–Lys–Lys–
- –Lys–Arg^{135}–Tyr–Gly–Gly–Phe–Met–Lys–Lys–
- –Lys–Arg^{185}–Tyr–Gly–Gly–Phe–Met–Arg–Gly–
- –Lys–Arg^{209}–Tyr–Gly–Gly–Phe–Met–Arg–Arg–
- –Lys–Arg^{229}–Tyr–Gly–Gly–Phe–Leu–Lys–Arg–
- –Lys–Arg^{260}–Tyr–Gly–Gly–Phe–Met–Arg–Phe–

Proenkephalin B
- –Lys–Arg^{174}–Tyr–Gly–Gly–Phe–Leu–Arg–Lys–
- –Lys–Arg^{208}–Tyr–Gly–Gly–Phe–Leu–Arg–Arg–
- –Lys–Arg^{227}–Tyr–Gly–Gly–Phe–Leu–Arg–Arg–

We previously developed a simple method for determining opioid peptides in rat brain by reversed-phase HPLC with on-line post-column fluorescence detection.[10] The method allowed only N-terminal tyrosine-containing peptides to be derived to fluorescent compounds with reagents of hydroxylamine, cobalt(II) and borate buffer (pH 8.5).

In the present study, the HPLC method was applied to the quantification of fragment peptides released from the opioid peptide precursors in rat brain tissues by tryptic cleavage. Since trypsin catalyzes the hydrolysis of peptide bonds at the carboxyl side of Arg and Lys, the following fragment peptides should be produced from the precursors: ME–Thr–Ser–Glu–Lys from proopiomeranocortin, ME–Lys, ME–Arg and LE–Lys from proenkephalin A, and LE–Arg from proenkephalin B (Table I). These fragment peptides were separated on a reversed phase column and on-line detected fluorometrically. The five fragment peptides were also identified with further enzymatic degradation using carboxypeptidase B. [D-Ala^2]ME was used as an internal standard. The distribution of the three opioid peptide precursors in the brain tissues was discussed.

Experimental

Chemicals and Solutions The following synthetic peptides were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification: Tyr–Gly, Tyr–Gly–Gly, Tyr–Phe, Tyr–Gly–Gly–Phe, ME, [D-Ala^2]ME, ME–Arg, ME–Lys, ME–NH_2, ME–Arg–Phe, ME–Arg–Gly–Leu, ME–Thr–Ser–Glu–Lys, α-endorphin (α-EDP, ME–Thr–Ser–Glu–Lys–Ser–Gln–Thr–Pro–Leu–Val–Thr), LE, LE–Arg, LE–Lys and LE–NH_2. The peptide solutions were prepared in water and stored at −80 °C. They were consumed within 3 weeks. Trypsin (EC 3.4.21.4, 12200 units/mg protein) from bovine pancreas, which has been added with L-1-tosylamido-2-phenylethylchloromethylketone, an inhibitor of chymotrypsin present as an impurity, and carboxypeptidase B (EC 3.4.17.2, 140 units/mg protein) from porcine pancreas were purchased from Sigma. Water was deionized and then distilled before use. The other chemicals were of the highest purity available.

Preparation of Rat Brain Protein and Tryptic Degradation Rat brain tissues were obtained from male Sprague–Dawley rats (220—280 g, 7 weeks). Brain areas were dissected according to the method previously described.[10] Each tissue (about 50 mg) was homogenized at 0—4 °C with 0.5 ml of 0.1 M HCl. The homogenate was centrifuged at 350 *g* for 20 min.

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The supernatant was added with 1 ml of acetone, and centrifuged again at 1500 *g* for 10 min. The precipitate (about 8-mg protein, which contained the precursors) was suspended in 350 μl of water. The suspension (50 μl) was used to determine protein by the Lowry method.[11] The suspension (300 μl) was mixed with 50 μl of 0.5 M Tris–HCl buffer (pH 7.8) containing 0.1 M $CaCl_2$ and 0.1 M NaCl, and 125 μl of trypsin (24000 unit/ml). When an inactivated trypsin was required for use, the trypsin solution was heated at 100 °C for 3 min. Finally, the volume of the enzyme reaction mixture was adjusted with water to 500 μl. The mixture was incubated at 37 °C for 60 min. Then 2.0 M $HClO_4$ (200 μl) was added to the mixture to stop the enzyme reaction. A portion (15 μl) of 5.0 nmol/ml [D-Ala²]ME (internal standard) was added to the enzyme reaction mixture. When calibration graphs were prepared, 10 μl of a solution of synthetic peptides (5—10 nmol/ml each) was added to the mixture. The reaction mixture was centrifuged at 1500 *g* for 5 min. The supernatant was transferred to a mini-cartridge packed with 0.2 g of Asahipregel TC18 (particle size, 40 μm; Asahi Chemical Ltd., Kawasaki, Japan). After loading the supernatant, the cartridge was washed twice with 2 ml of water. A fraction containing the fragment peptides was eluted with 1.5 ml of a mixture of acetonitrile and water (9 : 1, v/v). After evaporation *in vacuo* at approximately 30 °C, the residue was dissolved in water (total volume, 250 μl). The solution (60 μl) was diluted with water to 100 μl and subjected to HPLC. In the case of degradation with carboxypeptidase B, the solution was used without dilution.

Degradation of Fragment Peptides with Carboxypeptidase B A portion (60 μl) of the sample solution was mixed with 10 μl of carboxypeptidase B (2 units/ml) in 50 mM phosphate buffer (pH 7.8). The mixture was incubated at 37 °C for 30 min. The reaction mixture was heated at 100 °C for 3 min to stop the enzyme reaction. The mixture was centrifuged at 1500 *g* for 3 min and the whole supernatant was diluted with water to 100 μl and used for HPLC.

HPLC A Hitach 655A-12 high-pressure pump fitted with a Rheodyne 7125 syringe-loading sample injector (100 μl loop), programmed electronic valves for gradient elutions and a reversed phase column, Asahipak ODP-50 (150 × 6 mm i.d., particle size 5 μm, Asahi Chemical Ltd.) was used. Eluents A and B were prepared for gradient elution. They were mixtures of acetonitrile, 50 mM sodium borate buffer (pH 7.0) and water (1 : 4 : 15 and 3 : 1 : 1, v/v, respectively). The gradient profile of acetonitrile in the mobile phase is indicated by a dotted line in Fig. 1. The flow rate was 1.0 ml/min. The column temperature was ambient (24 ± 4 °C). A 0.3 M sodium borate buffer (pH 9.0) and 8 mM hydroxylamine oxalate solution containing 0.2 mM cobalt(II) acetate were added to the eluate stream by Hitachi 655 reagent-delivery pumps at a flow rate of 0.4 ml/min each. The mixture was passed through a reaction coil (10 m × 0.5 mm i.d.; stainless-steel tube) in an electronic heater at 100 °C. The eluate was monitored at 430 nm emission and at 330 nm excitation with a Hitachi F 1000 fluorescence spectrophotometer fitted with a 12-μl flow cell.

Results and Discussion

The conditions for the HPLC separation and fluorometric detection were almost the same as previously reported.[10] Figure 1 shows a chromatogram of a standard mixture (10 pmol each) of seventeen N-terminal Tyr-containing synthetic peptides. The five synthetic fragment peptides were well separated between the retention times of 13.8—29.2 min.

The investigation of the effect of trypsin concentration and incubation time on the production of the fragment peptides from the opioid peptide precursors revealed that maximum and constant production was achieved at trypsin concentrations between 200—500 μg (2400—6000 units)/tube, and that an incubation for 30 min or longer was necessary to obtain the maximal production (Fig. 2): incubation at 37 °C for 60 min in the presence of 250 μg trypsin/tube was used in the procedure.

The synthetic five fragment peptides were not degraded with trypsin under the same reaction conditions: the peak

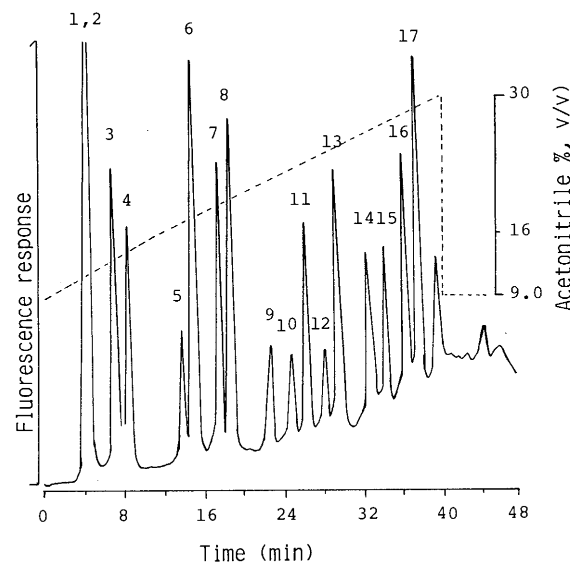
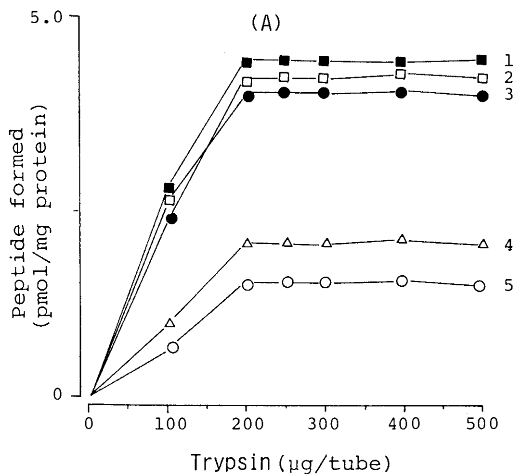


Fig. 1. Chromatogram Obtained with a Standard Mixture of Opioid Peptides and Related Peptides (10 pmol Each)

Peaks: 1, Tyr–Gly; 2, Tyr–Gly–Gly; 3, Tyr–Gly–Gly–Phe, 4, Tyr–Phe; 5, ME–Thr–Ser–Glu–Lys; 6, ME; 7, LE; 8, [D-Ala²]Me; 9, α-EDP; 10, ME-Lys; 11, ME-Arg; 12, LE-Lys; 13, LE-Arg; 14, ME–NH_2; 15, ME–Arg–Gly–Leu; 16, LE–NH_2; 17, ME–Arg–Phe.



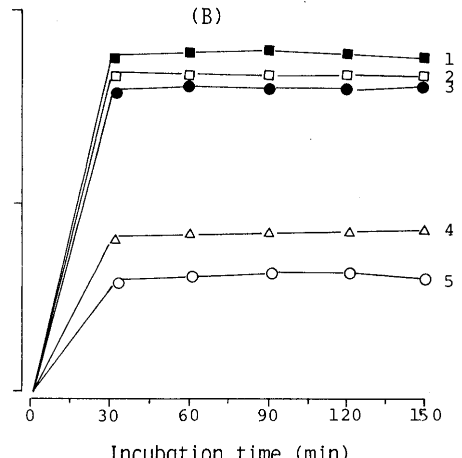


Fig. 2. Effect of (A) Trypsin Concentration and (B) Incubation Time (at 37°C) on the Formation of the Fragment Peptides from the Opioid Peptide Precursors in Striatum Tissue of Rat Brain

Curves: 1, ME–Lys; 2, ME–Arg; 3, ME–Thr–Ser–Glu–Lys; 4, LE–Lys; 5, LE–Arg.

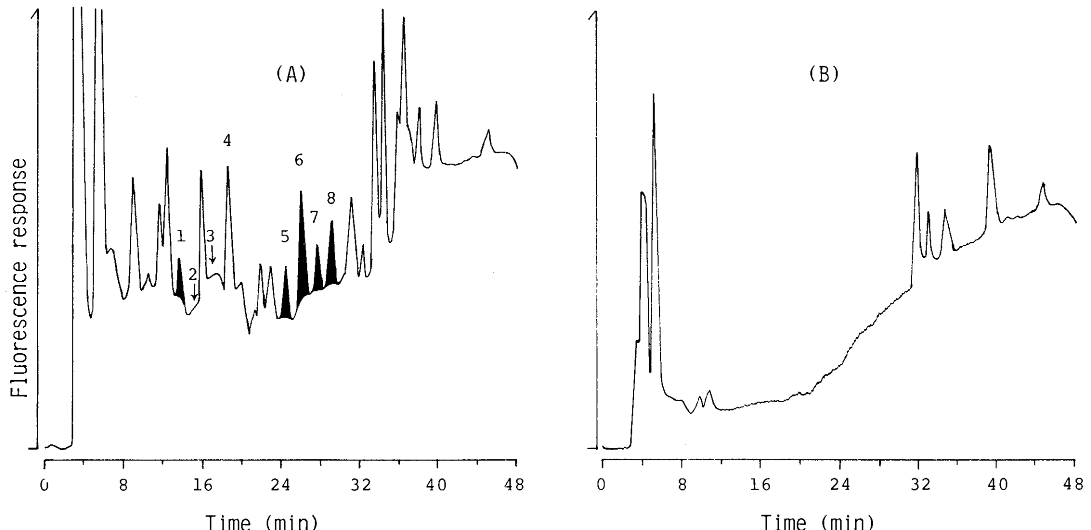


Fig. 3. Chromatograms Obtained with Striatum Protein after Degradation with (A) Trypsin and (B) Inactived Trypsin

Shaded peaks were due to the fragment peptides from the opioid peptide precursors. Peaks: 1, ME–Thr–Ser–Glu–Lys; 2, ME; 3, LE; 4, [D-Ala^2]ME (internal standard); 5, ME–Lys; 6, ME–Arg; 7, LE–Lys; 8, LE–Arg.

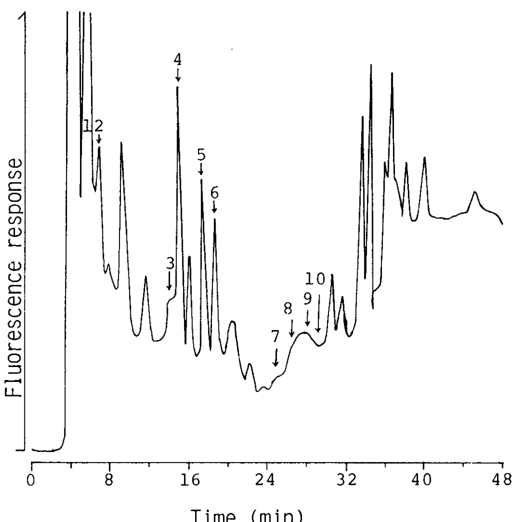


Fig. 4. Chromatogram Obtained with the Fragment Peptides (from Striatum Protein) Treated with Carboxypeptidase B

Peaks: 1, unknown compound(s) and ME–Thr–Ser—Glu; 2, Tyr–Gly–Gly–Phe; 3,ME–Thr–Ser–Glu–Lys; 4, ME; 5, LE; 6, [D-Ala^2]ME (internal standard); 7, ME–Lys; 8, ME-Arg; 9, LE–Lys; 10, LE–Arg.

height of the peptides was constant for the prolonged reaction period of 150 min.

Figure 3A depicts a chromatogram obtained with striatum protein after degradation with trypsin. Many peaks ascribable to N-terminal Tyr-containing peptides were detected: no peaks were observed when the brain protein was treated with inactivated trypsin (Fig. 3B). The shaded peaks corresponding to ME–Thr–Ser–Glu–Lys, ME–Lys, ME–Arg, LE–Lys and LE–Arg were identified on the basis of their retention times and also by co-chromatography with the synthetic peptides.

For additional identification, the same sample obtained after the tryptic digestion was further degraded by reaction with carboxypeptidase B. Carboxypeptidase B releases selectively a basic amino acid of Arg or Lys at the C-terminal end of the peptide. Therefore, the peaks corresponding to ME–Thr–Ser–Glu–Lys, ME–Lys, ME–Arg, LE–Lys and LE–Arg in Fig. 3A disappeared, and the peaks for ME and LE derived from ME–Lys, ME–Arg, LE–Lys and LE–Arg were newly produced (Fig. 4). The peak for ME–Thr–Ser–Glu, which should be produced from ME–Thr–Ser–Glu–Lys, could not be completely identified because the peak was overlapped with an unknown large peak (the retention times, 5.4 min) which increased significantly in height during the carboxypeptidase B hydrolysis of the trypsin-hydrolyzate in comparison with the other unknown peaks. Similar chromatogram patterns to those of Figs. 3 and 4 were obtained with other brain tissues such as hypothalamus, hippocampus and cortex. Further identification of the produced fragment peptides based on the analyses of amino acid composition and amino acid sequence, and also on liquid chromatography–mass spectrometry is in progress.

The recoveries of synthetic ME–Thr–Ser–Glu–Lys, ME–Lys, [D-Ala^2]ME, ME–Arg, LE–Lys and LE–Arg (50 pmol each) added to the reaction mixture of a striatum protein after digestion with trypsin were 50 ± 5, 70 ± 5, 85 ± 4, 80 ± 4, 65 ± 5 and $85 \pm 4\%$ (mean $\pm$ standard deviation, $n = 7$). The ratio of the recovery of each fragment peptide to that of the internal standard of [D-Ala^2]ME was always constant ($n = 7$). The calibration graphs for the five synthetic peptides added to the reaction mixture of each tissue protein were linear up to at least 15 pmol/mg protein and passed through the origin ($\gamma = 0.997 - 0.999$, $n = 3$ each plot). The detection limits (S/N = 3) for the peptides were 0.7—2.8 pmol per 100 μl injected. The precision was also examined using the striatum sample. The relative standard deviations ($n = 7$) were 3.4, 4.0, 3.0, 4.0 and 3.2% for 4.0 pmol ME–Thr–Ser–Glu–Lys, 4.4 pmol ME–Lys, 4.2 pmol ME–Arg, 2.1 pmol LE—Lys and 2.3 pmol LE–Arg per mg of protein, respectively.

The concentrations of the fragment peptides in the acetone-precipitated proteins from four regional tissues of rat brain were measured by the present method (Table II). The fragment peptides were found in all of the tissue proteins. ME–Lys, ME–Arg and LE–Lys appeared to be in the concentration ratio of approximately 3 : 3 : 1, which

TABLE II. Concentrations of the Fragment Peptides Produced from the Opioid Peptide Precursors in Rat Brain Tissues by Tryptic Degradation

Tissue	ME–Thr–Ser–Glu–Lys (from pro-opiomerano-cortin)	ME–Lys	ME–Arg	LE–Lys	LE–Arg (from proenke-phalin B)
		(from proenkephalin A)			
	(pmol/mg protein)				
Striatum	4.7	5.0	3.8	2.6	2.1
	4.0	4.4	4.2	2.1	1.6
	3.5	3.9	4.1	1.6	2.3
Hypothalamus	5.5	4.6	4.5	1.8	1.8
	4.0	4.2	4.8	2.1	1.9
	5.2	4.4	4.3	2.5	3.1
Hippocampus	3.9	2.0	1.4	0.5	1.1
	3.0	1.6	1.6	0.6	0.8
	2.8	1.7	2.2	0.7	0.8
Cortex	2.2	1.8	0.8	0.4	0.8
	2.3	1.9	1.2	0.5	0.6
	2.7	2.3	2.0	0.8	1.1

was coincided with the ratio of the fragment peptide moieties in proenkephalin A (Table I). The concentrations of all fragment peptides were higher in the striatum and hypothalamus tissues than in the hippocampus and cortex tissues. This agreed with the fact that opioid peptides such as ME and LE in the striatum and hypothalamus tissues were at higher concentrations than other brain tissues.[10] These observations suggest that the amounts of the fragment peptides are proportional to their precursor concentrations in the tissues: the concentrations of proenkephalin A, proopiomeranocortin and proenkephalin B distributed in the four tissues are higher in that order.

The recommended method has sufficient sensitivity and selectivity for the quantification of the fragment peptides released from the opioid peptide precursors in rat brain tissues by tryptic digestion, and may permit all the precursors to be estimated. Therefore, this method should be useful for the investigation of opioid peptides in brain tissues.

Acknowledgements We thank Asahi Chemical Ltd. for the generous gifts of the HPLC column and packing material for the mini-cartridge.

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Enzyme Labeling in Steroid Enzyme Immunoassays. Comparison of the *p*-Nitrophenyl Ester and *N*-Succinimidyl Ester Methods

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Enzyme labeling of steroids by the *p*-nitrophenyl ester method was investigated in comparison with the *N*-succinimidyl ester method. The active ester of a testosterone or 11-deoxycortisol derivative was treated with β-galactosidase and horseradish peroxidase to give labeled antigens. Various molar ratios of steroid to enzyme and pH conditions were tested. Satisfactory immunoreactivities with an anti-steroid antibody in each enzyme immunoassay system were obtained with the labeled antigens prepared at pH 8.5 by the use of molar ratios higher than 30. The enzyme labeling method should be useful in the case of polar steroids or drugs, since the *p*-nitrophenyl ester is relatively stable when compared with the *N*-succinimidyl ester.

Keywords enzyme immunoassay; steroid enzyme labeling; *p*-nitrophenyl ester methods; *N*-succinimidyl ester method; β-galactosidase; horseradish peroxidase; testosterone; 11- deoxycortisol

Enzyme-labeled antigens for use in the enzyme immunoassay of steroid hormones have usually been prepared by condensation of the carboxyl groups of a steroid hapten with the amino groups of lysine residues in an enzyme. The sensitivity and reproducibility of enzyme immunoassays are influenced by the coupling method. We have previously shown that the *N*-succinimidyl (NS) ester method is useful for alkaline phosphatase,[1] β-galactosidase (β-GAL),[2] horseradish peroxidase (HRP)[3] and glucose oxidase[4] labelings, as an alternative to the conventional methods, such as the mixed anhydride and carbodiimide methods. In general, the NS ester is stable when stored as a crystalline at 4 °C or in dioxane,[5] and hence, the active ester method is convenient for the reproducibility of enzyme immunoassay, especially, in systematic studies for obtaining a practical basis for selecting the enzyme, and on factors influencing the assay sensitivity. In the cases of some polar steroids or drugs, however, the preparation of the NS ester is troublesome because of its instability under purification and storing conditions. The activation of the carboxyl group as the *p*-nitrophenyl (*p*-NP) ester[6] may be the method of choice in such a case. This paper deals with the *p*-NP ester method in polyclonal testosterone and monoclonal 11-deoxycortisol assay systems using β-GAL and HRP as label enzymes, in comparison with the NS ester method.

Materials and Methods

Melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 in $CHCl_3$. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL JNM-FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Enzymic activity was measured with a Hitachi spectrophotometer 100-20.

Materials β-GAL (EC 3.2.1.23) from *Escherichia coli* (grade VI, 435 units/mg), and HRP (EC 1.11.1.7) (grade I-C, Reinheits-Zahl 3.15, 250 units/mg) were obtained from Sigma Chemical Co. (U.S.A.) and Toyobo Co. (Osaka), respectively. Anti-testosterone antiserum[7] and the monoclonal anti-11-deoxycortisol antibody[8] used were those reported in the previous papers. Normal rabbit serum and goat anti-rabbit immunoglobulin G (IgG) antiserum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo); normal mouse serum and rabbit anti-mouse IgG antiserum were from MBL Co. (Nagoya). 3,3′,5,5′-Tetramethylbenzidine and *o*-nitrophenyl β-D-galactopyranoside were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Nacalai Tesque Inc. (Kyoto), respectively.

Synthesis of Steroid NS Esters The NS esters of 4-hydroxytestosterone 4-hemiglutarate (T·HG),[7] 4-(carboxymethylthio)testosterone (T·CMT), 4-(2-carboxyethylthio)testosterone (T·CET),[9] and 4-(2-carboxyethylthio)-11-deoxycortisol (S·CET)[10] were prepared by the method described previously.[9] In short, a solution of the carboxylated steroids (0.5 mmol), *N*-hydroxysuccinimide (0.7 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (0.7 mmol) in 95% dioxane (2 ml) was stirred at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and passed through an Al_2O_3 layer. The filtrate was evaporated down to give the active ester.

General Procedure for the Preparation of Steroid *p*-NP Esters A solution of the carboxylated steroids (T·HG, T·CMT, T·CET, S·CET) (1 mmol), *p*-nitrophenol (1.3 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (1.8 mmol) in 95% dioxane (5 ml) was stirred overnight at room temperature. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The product was purified by chromatography on silica gel using hexane–AcOEt as an eluent, and recrystallized from an appropriate solvent.

T·HG *p*-NP Ester Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 2.4—2.9 (4H, 4-CO$\underline{CH_2}$CH$_2$$\underline{CH_2}$CO), 3.65 (1H, t, $J=8$ Hz, 17α-H), 7.30, 8.30 (each 2H, d, $J=9$ Hz, aromatic H).

T·CMT *p*-NP Ester Colorless needles from acetone–hexane. mp 135—137 °C. $[\alpha]_D^{20}$ +86° ($c=0.4$). *Anal.* Calcd for $C_{27}H_{33}NO_6S$: C, 64.91; H, 6.66; N, 2.80. Found: C, 64.88; H, 6.84; N, 2.74.

T·CET *p*-NP Ester Colorless needles from acetone–hexane. mp 112—114 °C. $[\alpha]_D^{20}$ +96° ($c=0.4$). *Anal.* Calcd for $C_{28}H_{35}NO_6S$: C, 65.48; H, 6.87; N, 2.73. Found: C, 65.28; H, 6.86; N, 2.64.

S·CET *p*-NP Ester Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.6—3.2 (4H, 4-SCH_2CH_2CO), 3.70 (1H, m, 6α-H), 4.30, 4.68 (each 1H, d, $J=20$ Hz, 21-H), 7.31, 8.28 (each 2H, d, $J=9$ Hz, aromatic H).

Buffer Solution 0.05 M Phosphate buffers (PBs), pH 5.4—8.0, were used in the study on stability and reactivity with glycine of the active ester, and in the enzyme labeling. 0.05 M Borate buffers (pH 8.5—9.0) were also employed in the labeling reaction. In the immunoassay, PB (pH 7.3) containing 0.1% gelatin and 0.9% NaCl was used.

Determination of Hydrolysis Rate of the Steroid NS and *p*-NP Esters PBs, pH 5.4—8.0 (0.2 ml), were each added to a solution of the CET NS or *p*-NP ester (0.05 mmol) in dioxane (0.1 ml), and the solution was allowed to stand at 26 °C. An aliquot of the reaction mixture was subjected to thin-layer chromatography (TLC) on silica gel using AcOEt–MeOH (4:1) as a developing solvent. The ratio of the remaining ester and hydrolyzate was determined with a Shimadzu dual-wavelength TLC scanner (240 nm); the rate of hydrolysis was calculated on the basis of the ratio values, since no side reaction occurred under the conditions tested.

Reaction of the Steroid NS and *p*-NP Esters with Glycine Glycine (0.2 mmol) in dioxane (0.1 ml) was added to a solution of T·CET NS or *p*-NP ester (0.05 mmol) in PBs, pH 5.4—8.0 (0.2 ml), and the solution was allowed to stand at 4 °C. An aliquot of the reaction mixture was subjected to TLC on silica gel using $CHCl_3$–MeOH–AcOH (40:5:0.4) as a developing solvent. The yield of the product was determined with the TLC scanner described above.

Enzyme Labeling of Steroid by the Active Ester Methods Dioxane solutions (0.1 ml) containing calculated amounts of the NS or *p*-NP ester

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corresponding to steroid/enzyme molar ratios of 10—100 (molecular weight of HRP,[11] 44000; β-GAl,[12] 465000) were each added to a solution of HRP or β-GAL (200 μg) in PB (pH 5.4—8.0, 0.2 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After addition of the corresponding PB (1.2 ml), the resulting solution was dialyzed against the cold buffer (2 l) for 2 d, then against PB, pH 7.3 (1 l), for 12 h. A 1 ml aliquot of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4 °C at a concentration of 100 μg/ml, adjusted with the assay buffer.

In the practical p-NP and NS ester methods, the β-GAL or HRP labeling was carried out with 1 mg of enzyme at pH 7.3—9.0 in dioxane (0.1 ml)–buffer (0.2 ml). The steroid/enzyme molar ratios examined were 10—100 in both labeliongs. The buffer used for dialysis was PB, pH 7.3.

Enzyme Immunoassay Procedure This was carried out in triplicate in glass test tubes (10 ml). The standard procedure for immunoreactivity of the testosterone labels is as follows: HRP- (2 ng) or β-GAL-labeled antigen (100 ng) in the assay buffer (0.1 ml) containing 0.5% normal rabbit serum was added to the diluted anti-testosterone antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h (first incubation). Goat anti-rabbit IgG antiserum (0.1 ml) diluted 1 : 30 with the assay buffer containing 0.3% ethylenediaminetetraacetic acid was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. After addition of the assay buffer (1.5 ml) the resulting solution was centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with the assay buffer (1.5 ml), and used for measurement of the enzymic activity (B_0).

In the monoclonal 11-deoxycortisol assay system,[8b)] the first incubation procedure using monoclonal anti-11-deoxycortisol antibody and enzyme-labeled 11-deoxycortisol was carried out in a manner similar to that described above. B/F separation was then carried out, using rabbit anti-mouse IgG antiserum diluted 1 : 120 with the assay buffer and 0.5% normal mouse serum.[13)]

In each system, the procedure without addition of the first antibody was carried out to provide non-specific binding values. An experiment using only the enzyme label was also carried out to obtain 100% enzymic activity (T).

Measurement of HRP Activity The immune precipitate or enzyme solution was diluted with 0.05 M acetate–citric acid buffer, pH 4.2 (1.8 ml), containing 0.42 mM 3,3′,5,5′-tetramethylbenzidine and 3% dimethylsulfoxide, vortex-mixed, and preincubated at 37 °C for 3 min. Hydrogen peroxide (0.02%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by the addition of 0.5 M H_2SO_4 (2 ml) and the absorbance was measured at 450 nm.

Measurement of β-GAL Activity The immune precipitate or enzyme solution was diluted with the assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol, vortex-mixed, and preincubated at 37 °C for 3 min. o-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in the assay buffer was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by the addition of 1 M Na_2CO_3 (2 ml) and the absorbance was measured at 420 nm.

Results and Discussion

The purpose of this work was to assess the p-NP ester method as an enzyme labeling technique, in comparison with the NS ester method. It has been suggested that the p-NP ester is more stable than the NS ester.[14)] Therefore, we compared the two active esters in the practical enzyme immunoassay procedure for the determination of steroids. The steroid derivatives used as haptens in this study were T·HG, T·CMT, and T·CET in the testosterone assay system. The aim of the employment of the various haptens was to examine the effect of the bridge length on the immunoreactivity of the labeled steroids prepared by the labeling methods. In order to test the applicability of the enzyme labeling at an higher pH, the monoclonal assay system for alkaline-sensitive 11-deoxycortisol was also studied. The p-NP and NS esters were prepared from the carboxylated derivatives by condensation with p-nitrophenol or N-hydroxysuccinimide in the presence of a water-soluble carbodiimide.

First, the stability of the p-NP and NS esters of CET was studied. The hydrolysis of the esters was investigated at pH 5.4—8.0 in 0.05 M phosphate buffer–dioxane. The rate of the reaction could be followed by the ratio of ultraviolet absorbance of the α,β-unsaturated ketone system in the ester and hydrolyzate at 240 nm on the TLC. The effect of pH on the stability of the esters of T·CET is shown in Fig. 1. It can be seen that the p-NP ester is more stable than the NS ester under the conditions tested. Both the esters were relatively stable at pH 5.4. The rate of hydrolysis increased with increasing pH value, where pseudo-first-order kinetics

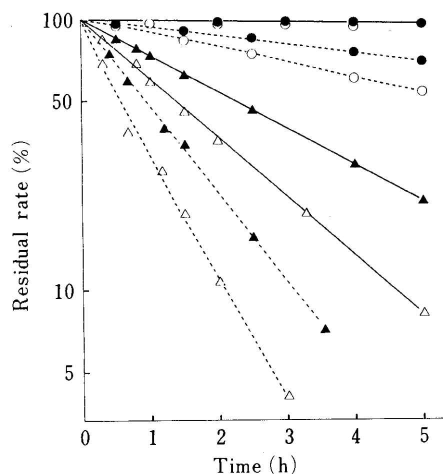

Fig. 1. Stabilities of the p-NP (—) and NS (---) Esters of T·CET at pH 5.4 (●), 6.2 (○), 7.3 (▲) or 8.0 (△) in Aqueous Medium

testosterone: R=H
T·CMT: R=SCH_2COOH
T·CET: R=$S(CH_2)_2COOH$
T·HG: R=$OCO(CH_2)_3COOH$

11-deoxycortisol: R=H
S·CET: R=$S(CH_2)_2COOH$

Chart 1

were observed. The reaction rate of the NS ester is 2—3 times as fast as that of the *p*-NP ester at pH 7.3—8.0. The half-time values of the hydrolysis at pH 7.3 were 55 and 135 min with the NS and *p*-NP esters, respectively. With the esters of S·CET, similar results were obtained.

Next, reactivities of the active esters of T·CET with the amino group of glycine were examined. The *p*-NP and NS esters were treated with 4 eq of glycine at various pHs in phosphate buffer–dioxane. The effect of pH on the reactivity is shown in Fig. 2. Distinct differences in reactivity can be observed. With the NS ester at pH 7.3, the formation of the product was efficient, resulting in almost 100% yield after the reaction time of 20 min. In the case of the *p*-NP ester, the yield was only 5% after 1 h at pH 7.3, despite the fact that a large amount of the starting compound still remained intact without being hydrolyzed. At pH 8.0, the reactivity of the *p*-NP ester was somewhat increased, but less than that of the NS ester at pH 6.2.

Immunoreactivities obtainable with the assay systems using the enzyme-labeled antigen prepared by the active ester method were then studied (Fig. 3). Two enzymes having a relatively high or low molecular weight, β-GAL and HRP, were employed as labels, since the molecular size of enzymes is a possible factor influencing the labeling and immune reactions. The enzyme labeling was first carried out at pH 5.4—8.0 by mixing the active ester with the enzyme in phosphate buffer–dioxane at 4 °C for 4 h. The active ester should react with free amino groups of these enzymes. Various molar ratios of the steroid to enzyme, ranging from 10 to 100, were used. The reaction mixtures were dialyzed against the buffer to remove the unreacted steroid and *p*-nitrophenol or *N*-hydroxysuccinimide. The loss of

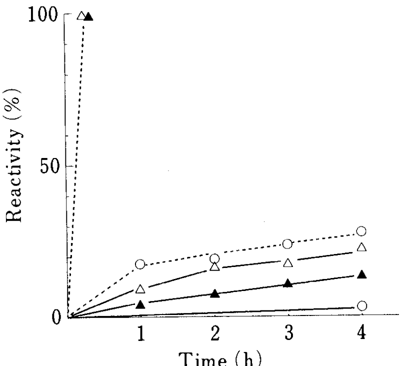

Fig. 2. Reactivities of the *p*-NP (—) and NS (---) Esters of T·CET with Glycine at pH 6.2 (○), 7.3 (▲) or 8.0 (△)

enzymic activity was less than 20% under the coupling conditions used. Determination of the number of steroid molecules incorporated per enzyme molecule was not essential for the present purpose, and hence, was not carried out. The anti-testosterone antiserum used in the enzyme immunoassay was that obtained in a rabbit by immunization with the conjugate of T·HG with bovine serum albumin (BSA).[7] In the system with the 11-deoxycortisol label, the monoclonal anti-steroid antibody was employed, which was secreted from the hybridoma derived from fusion of P3-NS1/1-Ag4-1 myeloma cells with spleen cells of BALB/c mice immunized with S·CET linked with BSA.[8a)] The bound and free enzyme-labeled antigens were separated by a double antibody method in both assay systems. The enzymic activity of immune precipitate was determined by colorimetric methods using 3,3′,5,5′-tetramethylbenzidine for HRP and *o*-nitrophenyl β-D-galactopyranoside for β-GAL as substrates.

The binding abilities of a fixed amount of the labeled antigens, in the testosterone system, were investigated at 1 : 500 dilution of the anti-testosterone antiserum. The results on the immunoreactivity of the enzyme-labeled T·HG, T·CMT and T·CET prepared at pH 7.3 by the active ester methods employing various steroid/enzyme molar ratios are shown in Fig. 4. In the assay systems with the β-GAL and HRP labels prepared by the NS ester method, the immunoreactivity (B_0/T) increased with increasing molar ratio in the labelings and satisfactory binding abilities were obtained with molar ratios higher than 20. On the other hand, the *p*-NP ester method gave much less reactive labels under the conditions tested in both enzyme systems. Although the difference between the two active ester methods in the effect of bridge length on the immunoreactivity was not significant in the present case, this factor may be important in some haptenic derivatives showing the steric hindrance. In Fig. 5, the results on the reactivity of the enzyme-labeled T·HG prepared at various pHs are shown. With the NS ester method, high reactivities were obtained at pH 8.0 as well as at pH 7.3 and lower reactivities were observed at pH 5.4—6.2. In contrast, the *p*-NP method did not give satisfactory results with respect to immunoreactivity, especially in the case of HRP labeling; this gave a label showing a very low reactivity even at pH 8.0. Similar results were obtained in the monoclonal 11-deoxycortisol assay system (data not shown). The results of the difference in reactivity between the HRP and β-GAL labels are reasonably related to the numbers of lysine residues in the enzyme molecules; the values have been

OH
O
OCO(CH$_2$)$_3$COOX → OCO(CH$_2$)$_3$CONH–enzyme

X = –C$_6$H$_4$–NO$_2$, –N(succinimide)

enzyme : β-GAL, HRP

Fig. 3. Enzyme Labeling of T·HG by the *p*-NP and NS Ester Methods

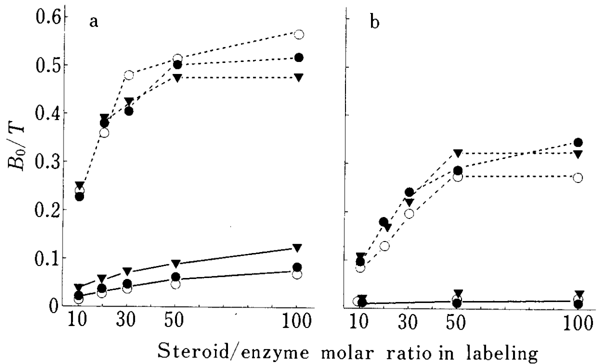

Fig. 4. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) T·HG (●), T·CMT (▼) and T·CET (○) Prepared at pH 7.3 by the *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

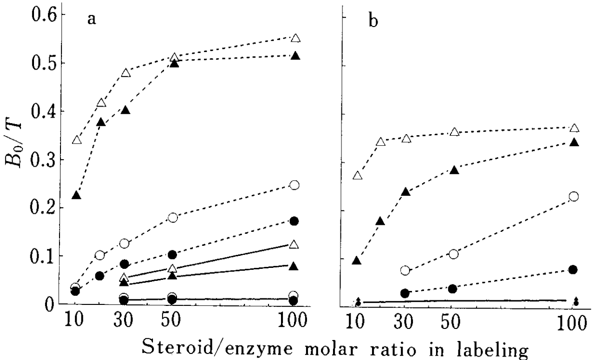

Fig. 5. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) T·HG Prepared at pH 5.4 (●), 6.2 (○), 7.3 (▲) or 8.0 (△) by the *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

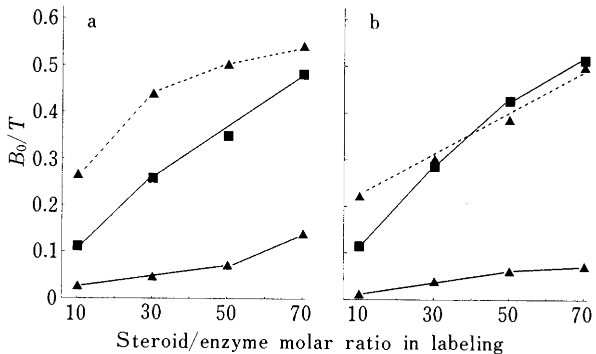

Fig. 6. Binding Abilities of the β-GAL- (a) and HRP-Labeled (b) S·CET Prepared at pH 7.3 (▲) or 8.5 (■) by the Practical *p*-NP (—) and NS (---) Ester Methods Using Various Steroid/Enzyme Molar Ratios

reported to be 6 for HRP[11] and 80 for β-GAL.[12]

Finally, based on the findings obtained above, we explored the practical enzyme labeling conditions in the *p*-NP ester method, using various alkaline conditions, steroid/enzyme molar ratios and reaction times. It was found, in both β-GAL and HRP labelings, that when the labeling was carried out at pH 8.5 for 4 h with the steroid/enzyme molar ratio of higher than 30, the *p*-NP ester method gave satisfactory results comparable to those obtained with an appropriate label prepared by the NS ester method. The results obtained with the monoclonal 11-deoxycortisol assay system are shown in Fig. 6. In testosterone and 11-deoxycortisol assay systems using the labeled antigen prepared at a molar ratio of 30, the desired

Table I. Enzyme Labeling Methods in Steroid Enzyme Immunoassay[a]

Method	Label enzyme	pH	Molar ratio[b]
NS	β-GAL	7.3	10—20
	HRP	7.3	10—60
p-NP	β-GAL	8.5	30—60
	HRP	8.5	30—60

a) Enzyme labeling with 1 mg of enzyme in dioxane (0.1 ml)-phosphate or borate buffer (0.2 ml) at 4 °C for 4 h. Final enzyme concentrations were 7.1 and 76 μM in the β-GAL and HRP labelings, respectively. *b*) Molar ratios of the steroid active ester to enzyme.

dose-response curves for the steroids with a high sensitivity equal to that in the previous works[1,5] could be obtained (data not shown). The use of a higher pH value than 9 resulted in the loss of β-GAL activity. With the HRP labeling, a somewhat higher pH can be used, but it is not effective in increasing the immunoreactivity; with the 11-deoxycortisol assay system, a marked decrease in the reactivity was observed. This is ascribable to the instability of the dihydroxy acetone chain in the 11-deoxycortisol molecule. The standard conditions in the NS[3] and *p*-NP ester methods for β-GAL and HRP labelings are listed in Table I.

In the present work, the *p*-NP ester was shown to be more stable than the NS ester under the neutral and alkaline conditions. The difference in stability of these esters was also observed in the purification by column chromatography on silica gel (data not shown). Thus, the *p*-NP ester method must be useful for the enzyme labeling of carboxylated haptens, especially with highly polar derivatives, when the isolation of active intermediates is needed. It should be noted that, with this active ester method, the enzyme and hapten itself used must be stable under alkaline conditions, if not, the employment of a large excess amount of the ester or a longer reaction time may be the method of choice for the preparation of a labeled antigen suitable for enzyme immunoassay.

The findings obtained here should be useful in the further development of enzyme immunoassay for steroid hormones and other haptenic compounds. We recommend molar ratios of 30—60 in both β-GAL and HRP labelings at pH 8.5 by the *p*-NP ester method (Table I). In the case of the NS ester method, the use of the molar ratios of 10—20 in the β-GAL labeling and 10—60 in the HRP labeling at pH 7.3 gives a good result with respect to the immunoassay sensitivity.[3] In general, however, it is desirable to estimate the degree of hapten substitution, since the labeling rate is influenced by various factors, such as pH, solvent volume, and reactivity of hapten derivatives. The present information should be helpful in enzyme labeling using alkaline phosphatase, glucose oxidase or other enzymes, and in the preparation of a hapten–carrier conjugate for use as an immunogen or the coated antigen in the microtiter plate immunoassay system.

Acknowledgement This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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Phytogrowth-Inhibitory Activities of Tropolone and Hinokitiol

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Tropolone (I) and hinokitiol (II) at concentrations of 50 ppm showed strong inhibitory activity on the growth of all plants examined. Compounds I and II completely inhibited the germination of the seeds of some plants at the same concentration. The phytogrowth-inhibitory activities of both compounds were higher than that of sodium 2,4-dichlorophenoxyacetate (2,4-D), which was used as a standard. Both compounds inhibited the growth of some plants even at a concentration as low as 10 ppm. On the other hand, unlike 2,4-D, I and II stimulated the growth of all plants at the low concentration of 1 ppm. At 7 d after treatment with I and II, the amount of chlorophyll in leaves of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS treated with both compounds was greatly decreased as compared with the control group.

Keywords tropolone; hinokitiol; tropolone-type compound; *Chamacyparis taiwanensis*; phytogrowth-inhibitory activity; *Brassica camperstris*; chlorophyll content; chlorophyll biosynthesis; inhibition

Hinokitiol (II, Chart 1), a tropolone (I, Chart 1)[1]-type compound, was isolated from the wood of *Chamacyparis taiwanensis* by Nozoe in 1936.[2] Since the discovery of this compound, various similar products (Chart 1) such as nootkatin,[3] α-thujaplicin[4] and γ-thujaplicin[4] have been isolated from higher plants. All of them inhibited the growth of wood-rotting fungi.[3,4] Among these compounds, there have been numerous reports on the biological activity of II because of its high yield. Namely, II was found to be effective as an antimicrobial agent[5-8] and a plant growth stimulator.[9] It has been reported that this compound prevented discoloration in fresh animal and plant food.[10] Compound I was also found to show antimicrobial activity.[11] However, no work has yet been done on the phytogrowth-inhibitory activity of the tropolone-type compounds.

Therefore, in this work, we extensively investigated the phytogrowth-inhibitory actions of I and II, and examined the chlorophyll content in leaves treated with both compounds in order to clarify the mechanism involved.

Materials and Methods

Chemicals Tropolone (I, Wako Pure Chemical Industries, Ltd.) and hinokitiol (II, Wako Pure Chemical Industries, Ltd.) were used for phytogrowth-inhibitory activity tests. Sodium 2,4-dichlorophenoxyacetate (2,4-D, Tokyo Kasei Co., Ltd.) was used as a positive control.

Plants The eight plants listed in Table I were used for the inhibitory activity tests.

Phytogrowth-Inhibitory Activity Test[12] Aliquots (1 ml) of dimethylsulfoxide (DMSO) of I, II and 2,4-D were each diluted in 100 ml of sterilized agar (0.8%, Difco) to concentrations of 1, 10 and 50 ppm. An agar-containing chemical or DMSO alone (control) was poured into a 500 ml sterilized biopot. Then, 20 seeds of each plant sterilized with 70% EtOH and 1% NaClO were put on the agar and left for 7 d under 9000 lux illumination. The lengths of the roots were measured and averaged. The phytogrowth-inhibitory activity was expressed as the ratio of the length of roots treated with the chemicals to that of the control (1.00).

Quantitative Analysis of Chlorophyll in the Leaves of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS The A.O.A.C. method[13] was used to determine the amounts of chlorophyll in the groups treated with I, II and 2,4-D as well as the control group at 7 d after treatment.

tropolone (I) hinokitiol (II) nootkatin

α-thujaplicin γ-thujaplicin

Chart 1

Results

Inhibitory Effects of Tropolone (I) and Hinokitiol (II) on Plant Growth Inhibitory effects of I and II were investigated using eight kinds of plants. As shown in Table I, both compounds strongly inhibited the growth of all of the plants examined at the concentration of 50 ppm. The inhibitory activities of both compounds at the same concentration were higher than that of 2,4-D, used as a standard. In particular, I completely inhibited the germination of the seeds of *Sesamum indicum* L. and *Lactuca sativa* L. var. *crispa* L., while II also exhibited inhibition of the germination of the seeds of *Lactuca sativa* L. var. *crispa* L., *Brassica chinensis* and *B. camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS at the same concentration. Compound I showed an inhibitory effect on all plants except for *Raphanus sativus* L. var. *acanthiformis* MAKINO even at the low concentration of 10 ppm. Compound II also inhibited the growth of half the number of plants examined at the same concentration. However, the inhibitory activities of both compounds were lower than that of 2,4-D. On the other hand, compounds I and II stimulated the growth of all of the plants examined at the low concentration of 1 ppm. In particular, both compounds strongly stimulated the growth of *Lactuca sativa* L. var. *crispa* L. at the same concentration.

Toxicity of Tropolone (I) and Hinokitiol (II) to *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS. Observation of the Growth Process After the strong phytogrowth-inhibitory actions of I and II had been confirmed, we investigated how the growth process of *R. camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS was affected by treatment with both compounds. As shown in Figs. 1 and 2, at 1 d

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TABLE I. Inhibitory Effects of Tropolone (I) and Hinokitiol (II) on Plant Growth

Plant	Growth (ratio)[a]								
	Troplone (I) (ppm)			Hinokitiol (II) (ppm)			2,4-D[b] (ppm)		
	50	10	1	50	10	1	50	10	1
Brassica rapa L.	0.020	0.442	1.247	0.006	0.924	1.104	0.076	0.072	0.100
Sesamum indicum L.	—	0.047	1.128	0.036	0.663	1.232	0.020	0.041	0.258
Raphanus sativus L. var *acanthiformis* MAKINO	0.023	1.132	1.642	0.025	1.064	1.745	0.019	0.071	0.115
Cucumis sativus L.	0.034	0.737	1.044	0.035	1.090	1.097	0.107	0.156	0.247
Lactuca sativa L. var. *crispa* L.	—	0.712	12.729	—	—	7.720	—	—	0.022
Glycine max MERR	0.398	0.477	0.818	0.165	1.077	1.350	0.088	0.173	0.171
Brassica chinensis	0.006	0.495	1.935	—	1.164	1.701	0.050	0.064	0.081
Brassica camperstris L. subsp. *rapa*. HOOK. f. *et* ANDERS	0.007	0.211	1.117	—	0.305	0.962	0.054	0.074	0.098

a) Growth in control experiments after 7 d was taken as 1.00. Quantity of light: 9000 lux. Experiment size: 20 seeds/group, 2 groups. —: no germination. *b*) Sodium 2,4-dichlorophenoxyacetate.

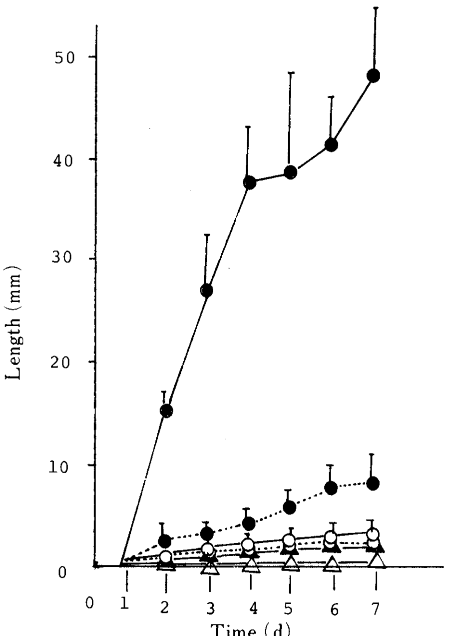

Fig. 1. Inhibitory Effect of Tropolone (I) on the Growth of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS

—△—, 50 ppm; —▲—, 40 ppm; --○--, 30 ppm; —○—, 20 ppm; --●--, 10 ppm; —●—, control. Each value represents the mean ± S.D. ($n=20$). Experimental size: 20 seeds/group, 2 groups.

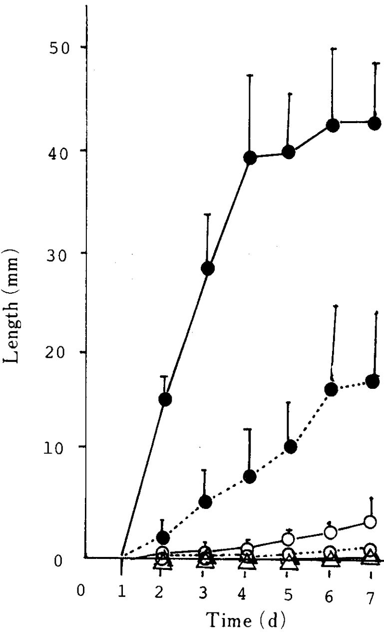

Fig. 2. Inhibitory Effect of Hinokitiol (II) on the Growth of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS

—△—, 50 ppm; —▲—, 40 ppm; --○--, 30 ppm; —○—, 20 ppm; --●--, 10 ppm; —●—, control. Each value represents the mean ± S.D. ($n=20$). Experimental size: 20 seeds/group, 2 groups.

after germination, growth differences could be detected between the treated groups and the control group. Thereafter, an inhibitory effect on growth, which increased with the passage of time, was recognized in very treated group.

Figures 3 and 4 show the differences in growth between groups treated with both compounds and the control group at 7 d after germination. It was found that all groups treated with I and II showed strong growth inhibition compared with the control group.

Changes of Amounts of Chlorophyll in Leaves Direct observation revealed that the surface of leaves turned white in every treated group. The leaves of groups treated with 10, 20 and 30 ppm of I and II were investigated to determine the amount of chlorophyll at 7 d after germination. As shown in Table II, the amounts decreased in groups treated

TABLE II. Chlorophyll Contents of *Brassica camperstris* L. subsp. HOOK. f. *et* ANDERS at 7 d after Treatment with Tropolone (I) and Hinokitiol (II)

Chemical	Concentration (ppm)	Total chlorophyll (%)[a]	Chlorophyll a (%)	Chlorophyll b (%)
Tropolone (I)	10	3.628	2.664	0.967
	20	3.283	2.451	0.934
	30	0.938	0.654	0.284
Hinokitiol (II)	10	3.696	2.661	1.037
	20	2.626	1.920	0.708
	30	0.327	0.190	0.137
2,4-D[b]	10	5.770	4.319	1.454
	20	5.565	4.082	1.487
	30	3.514	2.577	0.939
Control		11.995	8.330	3.674

a) % (wet wt.). Analytical method: A.O.A.C. method. Observation time; 7 d. Temperature: 27 °C. Illumination: 9000 lux. *b*) Sodium 2,4-dichlorophenoxyacetate.

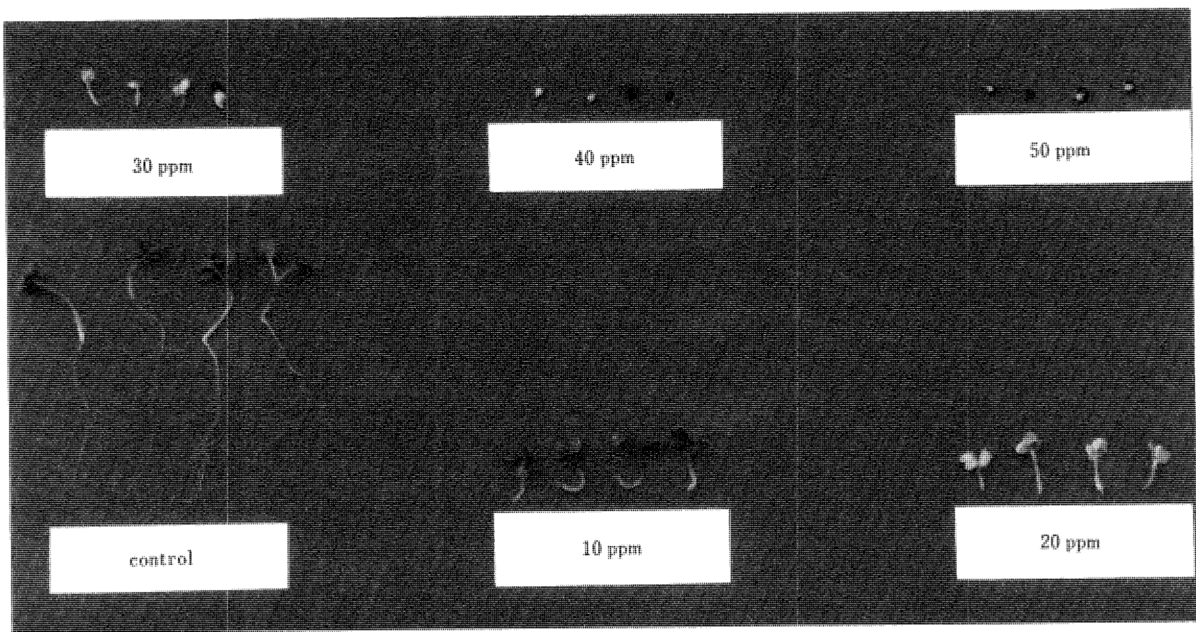

Fig. 3. Inhibitory Effect of Tropolone (I) on the Growth of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS at 7 d after Treatment

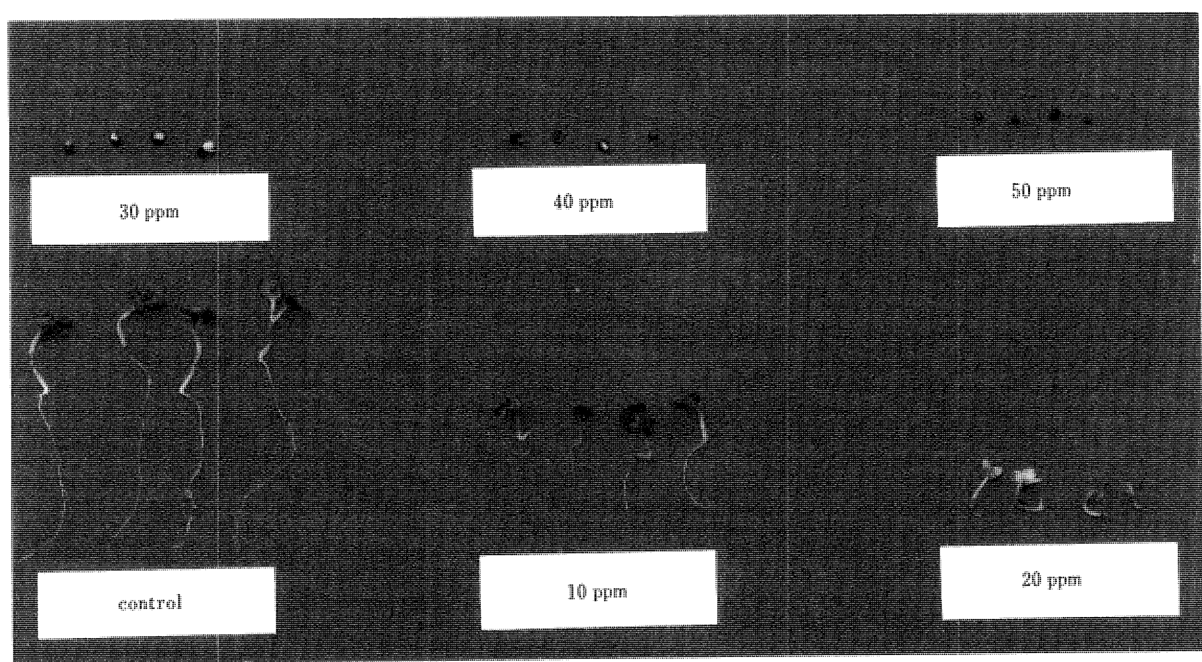

Fig. 4. Inhibitory Effect of Hinokitiol (II) on the Growth of *Brassica camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS at 7 d after Treatment

with both compounds as compared with the control group. In particular, the amounts were markedly decreased in groups treated with 30 ppm of I and II. The inhibitory effect of both compounds on chlorophyll biosynthesis was stronger than that of 2,4-D, used as a standard.

Discussion

Tropolone (I) and hinokitiol (II) were found to show strong phytogrowth-inhibitory activity at the concentration of 50 ppm (Table I). The inhibitory activities of both compounds at the same concentration were higher than that of sodium 2,4-D, used as a standard. The results indicate that the inhibitory activity of both compounds on plant growth was relatively strong at a high concentration (Table I, Figs. 1 and 2). However, the phytogrowth-inhibitory activity of both compounds at the concentration of 10 ppm was lower than that of 2,4-D. On the other hand, I and II stimulated the growth of all of the plants examined at the low concentration of 1 ppm. In particular, both compounds stimulated the growth of *Lactuca sativa* L. var. *crispa* at the same concentration (Table I, the growth (ratio) of I was twelve times that of the control group, while the growth (ratio) of II was seven times that of the control group). The phenomena of the growth between inhibition and stimulation at the different concentrations (1 and 10 ppm) is of considerable interest. However, the reason for this is not clear at present. As regards the action of II on plants, Kafuku[9] already reported that this compound was effective

as a plant growth stimulator. Our present results regarding the low concentration of 1 ppm are in accord with the report of Kafuku,[9] in which no detailed data on plant growth were described. However, the inhibitory effects of I and II on plants growth are reported for the first time in this paper.

Compounds I and II were found to inhibit chlorophyll biosynthesis in leaves of *B. camperstris* L. subsp. *rapa*. HOOK. f. *et* ANDERS at 7 d after treatment (Table II, Figs. 3 and 4). The findings suggest that chlorophyll inhibition by both compounds was relatively strong. On the other hand, the amount of chlorophyll in groups treated with both compounds at concentrations less than 1 ppm was almost equal to that in control group (data not shown). Considering that although the inhibitory effect of 2,4-D at the concentration of 10 ppm was stronger than that of I and II, and that of the inhibitory activity of the former on chlorophyll biosynthesis at the same concentration was lower than that of both compounds, it is premature to conclude that the primary site of action of both compounds is chlorophyll inhibition. The relationship between phyto-growth-inhibitory activity and chlorophyll inhibition of both compounds should be investigated. Coumarins,[14–16] δ-aminolevulinate,[17,18] kinetin[19] and fusicoccin[20] have already been isolated from higher plants as chlorophyll inhibitors. However, the inhibitory effects of I and II on chlorophyll biosynthesis are reported for the first time in this paper.

Acknowledgment The authors wish to express their deep gratitude to Professor G. Kusano Ph. D., of Osaka University of Pharmaceutical Sciences, for his valuable advice during this project.

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Differential Effects on Fatty Acid Compositions in the Liver Microsomes of Thyroidectomized or Streptozocin Induced Diabetic Rats

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The purpose of the present study was to locate a controversial site and to make generalizations about the effects of thyroidectomy (Tx) and streptozocin (STZ) on the distribution pattern of an individual fatty acid in microsomal fractions of the animals thus treated. The results obtained were compared with the reported data. The effects of Tx on $C_{18:1}$, $C_{18:2}$, and all detectable C_{20} and C_{22} species harmonized well within each species; however the effects of Tx on C_{16} species and $C_{18:0}$ varied within each species. Meanwhile, all the effects of STZ were identical within the species, but were often in opposite directions between two adjacent species; *e.g.* $C_{18:0}$ and $C_{18:1}$. These findings strongly indicate that desaturation and elongation sites were independently affected by either Tx or STZ. The comparison suggested that controversial effects appeared in the distribution proper to species C_{18}. Therefore, delta 9-desaturase activity in the microsomal fractions was measured, using stearoyl coenzyme A (CoA) as substrate, resulting in some partial reduction in Tx, but complete suppression in STZ-treated animals. The total contents of phospholipid and cholesterol in the microsomes were also measured. Results showed a significant increase in microsomes within the STZ-group, but almost no change in the Tx-group, indicating that the changes in an individual fatty acid component and in the total fatty acids do not always take place in parallel.

Keywords fatty acid composition; lipid composition; thyroidectomy; streptozocin; desaturase; stearoyl CoA

Hormones[1] regulate the fluidity of biomembranes by controlling the compositions of fatty acids, phospholipid constituents and cholesterol. Some specific fatty acids, intermediates and metabolic pathways in particular organs are affected by experimentally induced hyperthyroidism[2,3] or hypothyroidism[4-6] and diabetes.[7-11] Haas and Carter[5] and Hoch *et al.*[6] reported the changes in fatty acid composition in liver microsomes of thyroidectomized (Tx) rats, whilst Faas and Carter[10] and Huang *et al.*[11] studied the changes in streptozocin (STZ)-induced diabetic rats. When these analytical data on individual fatty acid compositions are carefully compared with each other between Tx-rats and STZ-diabetic rats, the increase or decrease in a particular component always shows good harmony, whereas, the direction of changes in other components never show harmony between the two groups. Such discrepancies may, in some cases, be within a margin of experimental error,[1] but may, in other cases, reflect a complicated physiological condition due to Tx- or STZ-treatment.

In the present study, we were interested in determining where the distribution profiles of fatty acid compositions show a coinsidence in such different treatments as Tx and STZ-administration and where the discrepancy is found between the two treatments. Thus, we examined the distribution pattern of fatty acids in liver microsomal fractions obtained from Tx-rats and STZ-induced diabetic rats and compared the profiles of fatty acid distribution caused by these treatments. Consequently, we found that a great similarity in fatty acid distribution between Tx-rats and STZ-diabetic rats was found in multiunsaturated fatty acids with higher carbon numbers than C_{18} and most discrepancies were found with delta 9-desaturase related fatty acid components.

Materials and Methods

Chemicals Standard fatty acids and their methyl esters were purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.) and Wako Chemical Co., Ltd. (Osaka, Japan). Coenzyme A (CoA), streptozocin, thyroxine (T_4) and triiodothyronine (T_3) were from Sigma Chemical Co. Thin layer chromatography plate was DC-Fertigplatterkiesel 60, a product of Merck Co., Ltd. (Darmstadt, Germany). Other chemicals were of special reagent grade available from local suppliers.

Animal Experiment and Lipid Extraction Male Wistar rats were bred, maintained on regular pellet chow (Oriental Yeast Co., Ltd., Tokyo, Japan, type MF) in a 12 h light–dark cycle in an air-conditioned house, and given tap water *ad libitum*. Under anesthesia with sodium isomytal, thyroidectomy was carried out on 3 week old male rats. The animals operated on were supplied with water containing 0.1% $CaCl_2$ to prevent parathyroprivic tetany. Hypothyroidism was stabilized for two weeks after the operation. Diabetes was induced into 5 week old male rats by a single i.v. injection of 60 mg/kg of STZ. Diabetic animals possessed a blood sugar level of above 400 mg/dl and were used at least two weeks after diabetic conditions stabilized.

Microsomal fraction was obtained by the method of Hogeboom.[12] Further purification of microsomes was carried out by Brenner and Peluffo's method.[13] The purified microsomal fraction showed the highest glucose 6-phosphatase activity of all the subcellular fractions, and no detectable activity of succinate dehydrogenase.

Lipid extraction was carried out by the method of Bligh and Dyer[14] with a micronized scale. Saponification, methylesterification and analysis by gas–liquid chromatography took place according to Hoch *et al.*[6] Phospholipid and cholesterol determinations were carried out on ethanol–ether (3 : 1, v/v) extracts of the microsomal preparations. Phosphorus content and cholesterol content were determined according to Chen *et al.*[15] and Rudel *et al.*,[16] respectively.

Other Measurements Delta 9-desaturase activity was measured by the method described in a previous paper.[17] Serum T_4 and T_3 were determined by SPAC T_4 and T_3 kits (Mallincrodt, St. Louis, Mo. U.S.A.). The serum insulin level was measured by an Eiken insulin kit (Tokyo, Japan). Protein was measured by Lowry's method.[18]

Statistical Analysis Statistical analyses were performed according to Student's *t*-test.

Results

Affirmation of the Effects of Thyroidectomy and STZ-Administration To assess hypothyroidism and diabetes caused by Tx and STZ-administration, respectively, changes in serum hormone concentration were measured. Table I shows the serum T_4, T_3 and insulin levels of rats treated with either Tx or STZ. Serum T_4 was significantly reduced by Tx: it was one-tenth of that in the control. T_3 was also reduced by 30% of the control. The serum insulin level in the Tx-group was reduced but the values varied between individual animals. Meanwhile, diabetes induced by STZ

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was confirmed by changes in serum insulin and blood glucose levels; in the diabetic group serum insulin fell to 37% of the control level, and the serum glucose level was over 400 mg/dl on average (data not shown). Lower serum T_3 and T_4 levels than those in the control were also observed in the diabetic group.

Alteration in Fatty Acid Composition Table II shows the result of fatty acid composition analyses of the liver microsomes of Tx- and diabetic groups. The composition of the fatty acids was altered in hypothyroidism or in diabetes. In the Tx-group, palmitic acid (16:0) and palmitoleic acid (16:1) were almost unchanged. Stearic acid (18:0) was increased but oleic (18:1) or linoleic acid (18:2) did not change, regardless of their origins, either *de novo* synthesis or dietary intake. Eicosatrienoic acid (20:3) was significantly increased, whereas arachidonic acid (20:4) was significantly reduced. A minor component of $C_{22:5}$ was almost unchanged and docosahexaenoic acid (22:6) increased slightly. In the diabetic group, palmitic acid and palmitoleic acid were reduced. Stearic acid increased. Oleic acid decreased but linolenic acid increased. Eicosatrienoic acid increased significantly but arachidonic acid decreased. A minor component of $C_{22:5}$ was reduced. Docosahexaenoic acid increased. Thus, in species C_{16} and C_{18}, the effects of Tx and STZ-administration on their fatty acid composition were quite in contrast, except for $C_{18:0}$, where both the treatments resulted in an increase with statistical significance. Tx and STZ-administration were in accordance with their effects on the composition within the representatives of C_{20}; both treatments resulted in an increase in $C_{20:3}$ but a decrease in $C_{20:4}$. On the other hand, in species C_{22}, Tx caused a reduction in $C_{22:5}$ but STZ did not. In turn, Tx did not affect the content of $C_{22:6}$ but STZ increased it significantly.

In Table III, the ratios of 16:1 to 16:0 and the like reflect the effects of Tx and STZ-induced diabetes on various desaturation processes (designated the desaturation index). Tx stimulated a conversion of $C_{16:0}$ to $C_{16:1}$ and significantly inhibited the conversion of $C_{20:3}$ to $C_{20:4}$. STZ did not affect the ratio of $C_{16:1}$ to $C_{16:0}$ but strongly lowered that of $C_{18:1}$ to $C_{18:0}$. The ratio of $C_{18:2}$ to $C_{18:1}$ was unaffected by Tx but was elevated by STZ. However, this ratio may not represent the state of the desaturation because of a lack of the process in animals.[1] STZ, like Tx, inhibited desaturation in species C_{20} and caused an increase in the ratio of $C_{22:6}$ to $C_{22:5}$. Table III also includes information about Tx and STZ effects on elongation of fatty acids in the ratios of C_{18} to C_{16} and the like (designated the elongation index of individual fatty acids); for example, C_{18}/C_{16} indicates a ratio of the sum of $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ to that of $C_{16:0}$ and $C_{16:1}$. Either Tx or STZ-treatment heightened the ratios of C_{18}/C_{16} and C_{22}/C_{20}, but lowered the ratio of C_{20}/C_{18}.

Fluidity of membrane is dependent upon certain amounts of unsaturated fatty acids, cholesterol and phospholipids. Since the distribution of major fatty acid components in the microsomal fraction were affected by Tx and STZ-administration, the total contents of phospholipids and cholesterol in the fraction were measured. Figures 1a and 1b show the findings of phospholipid and cholesterol contents in the microsomes, respectively. Phospholipids were unchanged by Tx, but were significantly increased by

TABLE I. Serum Hormone Levels in Hypothyroidism (Tx) and Diabetes (STZ)

Treatment	Insulin (μU/ml)	T_4 (μg/dl)	T_3 (ng/dl)
Control ($n=7$)	33.56 ± 9.20	4.80 ± 0.87	110.3 ± 18.0
Tx ($n=7$)	21.51 ± 20.17	0.54 ± 0.19$^{b)}$	78.8 ± 9.0$^{a)}$
STZ ($n=6$)	12.26 ± 3.32$^{b)}$	1.93 ± 0.54$^{b)}$	76.7 ± 5.3$^{a)}$

Values represent the mean ± S.D. *a)* $p<0.01$, *b)* $p<0.001$.

TABLE II. Major Fatty Acid Distribution of Microsomal Fractions in Control, Hypothyroidal (Tx) and Diabetic (STZ) Rat Livers

Treatment	Major fatty acids								
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:5	22:6
Control	24.87 ± 1.87	0.98 ± 0.48	19.72 ± 1.69	8.15 ± 0.97	16.57 ± 2.35	0.36 ± 0.09	20.87 ± 2.30	1.52 ± 0.48	7.00 ± 1.02
Tx	24.08 ± 2.32	1.51 ± 0.47	22.32 ± 1.25$^{b)}$	8.33 ± 1.30	17.04 ± 2.12	2.00 ± 0.75$^{c)}$	13.40 ± 3.24$^{c)}$	2.00 ± 0.54	9.02 ± 1.00
STZ	21.04 ± 1.37$^{b)}$	0.58 ± 0.17$^{a)}$	22.88 ± 0.86$^{c)}$	6.26 ± 0.92$^{b)}$	21.45 ± 2.66$^{b)}$	1.71 ± 0.36$^{c)}$	14.11 ± 3.13$^{c)}$	1.16 ± 0.21$^{b)}$	10.83 ± 2.56$^{a)}$

Values represent percent by weight of total fatty acids. The mean ± S.D. of 10 animals in control and Tx-group, and 7 animals in STZ-group. *a)* $p<0.05$, *b)* $p<0.01$, *c)* $p<0.001$.

TABLE III. Desaturation and Elongation Indices of Fatty Acids of Microsomal Fractions in Control, Hypothyroidal (Tx) and Diabetic (STZ) Rat Livers

Treatment	Desaturation index					Elongation index		
	16:1/16:0	18:1/18:0	18:2/18:1$^{a)}$	20:4/20:3	22:6/22:5	C_{18}/C_{16}	C_{20}/C_{18}	C_{22}/C_{20}
Control	0.039 ± 0.018	0.419 ± 0.079	2.043 ± 0.259	60.84 ± 16.47	4.99 ± 1.74	1.674 ± 0.189	0.490 ± 0.057	0.404 ± 0.072
Tx	0.063 ± 0.017$^{b)}$	0.376 ± 0.072	2.12 ± 0.17	7.21 ± 2.63$^{d)}$	4.73 ± 1.00	1.909 ± 0.177$^{b)}$	0.321 ± 0.070$^{d)}$	0.698 ± 0.176$^{c)}$
STZ	0.027 ± 0.010	0.275 ± 0.049$^{d)}$	3.461 ± 0.477$^{d)}$	8.62 ± 2.80$^{d)}$	9.36 ± 1.45$^{d)}$	2.365 ± 0.169$^{d)}$	0.305 ± 0.074$^{d)}$	0.833 ± 0.331$^{b)}$

Figures like 16:1/16:0 stand for "desaturation index," representing the ratios of concentration of corresponding fatty acids. C_{18}/C_{16} and the like stand for "elongation index," representing the ratios of the sum of all detectable fatty acids with the same carbon number to the sum of fatty acids with another carbon number. *a)* The ratio of $C_{18:2}/C_{18:1}$ may not directly represent the desaturase activity because of the lack of the pathway in *de novo* synthesis. Other explanations are the same as those in Table II. *b)* $p<0.05$, *c)* $p<0.01$, *d)* $p<0.001$.

Table IV. Comparison of the Effects of Hypothyroidism (Tx) and Diabetes (STZ) on Fatty Acid Distribution of Microsomes in Rat Livers, as Appearing in References

Reference	Source	Major fatty acids								
		16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:5	22:6
Tx-group										
Faas and Carter (5)	Liver Mc	±(↑)	±(↓)	±(↓)	±(↑)	↑	↑	↓	↑	±(↑)
Hoch *et al.* (6)	Liver Mc	±(↑)		±(↑)	±(↑)	↑	↑	↓		±(↑)
Nishida *et al.*	Liver Mc	±(↓)	↑	↑	±(↑)	±(↑)	↑	↓	±(↑)	↑
STZ-group										
Faas and Carter (10)	Liver Mc	±(↓)	↓	±(↑)	↓	↑	±(↑)	↓		
Faas and Carter (10)	Liver Mc	↓	↓	±(↑)	↓	↑	±(↑)	↓		↑
Nishida *et al.*	Liver Mc	↓	↓	↑	↓	↑	↑	↓	↓	↑

Arrows without parenthesis indicate the direction of changes with statistical significance, whereas ± represents changes without such significance. Arrows in parenthesis show the tendency of the changes. Mc: microsomes.

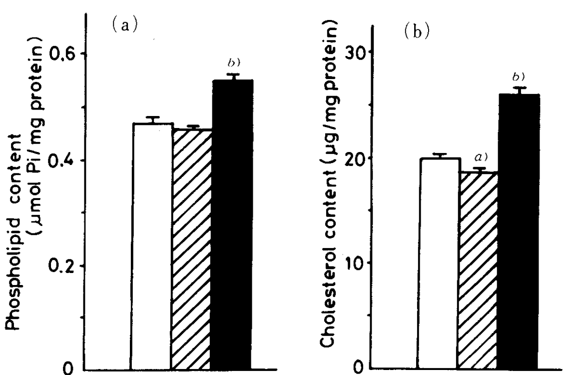

Fig. 1. Effect of Hypothyroidism (Tx) and Diabetes (STZ) on Phospholipid (a) and Cholesterol (b) Contents in Microsomes of Rat Livers

Values represent the mean ± S.D. of number of animals as shown in parenthesis. *a)* $p<0.05$, *b)* $p<0.001$. ▭, control ($n=10$); ▨, Tx ($n=10$); ■, STZ ($n=7$).

Table V. Delta 9-Desaturase Activity in Hypothyroidism (Tx) and Diabetes (STZ)

Treatment	Delta 9-desaturase activity (nmol oleate/min/mg protein)
Control ($n=10$)	0.194 ± 0.006
Tx ($n=10$)	$0.041 \pm 0.007^{a)}$
STZ ($n=7$)	ND

Values represent the mean ± S.D. *a)* $p<0.001$. ND: not detectable.

STZ administration. Whereas Tx slightly reduced the microsomal cholesterol, STZ treatment resulted in a rise in such content. Although details have not presented, the changes in phospholipids and cholesterol were not due to a reduction in the tissue protein content but to an *in situ* change in both lipids.

Comparison of the Fatty Acid Distribution in the Present Study with Those in Reference and Changes in Delta 9-Desaturase Activity When the data on individual fatty acid component distribution in hypothyroidism and diabetes which emerged from Table II were re-arranged in Table IV to show the direction of changes in the fatty acid distribution and to compare the present information with that obtained from other laboratories, the general trends in the multiunsaturated components of $C_{18:2}$, $C_{20:3}$ and $C_{20:4}$ obtained from either the Tx-group or the STZ-treated group matched well with each other, but the changes in the contents of the C_{16} and C_{18} specimens were often in opposite directions between the two treatments or they were inconsistent among the researchers; therefore, delta 9-desaturase activity was measured to investigate whether the enzyme activity correlates with the changes in the fatty acid distribution. When stearoyl CoA was used as a substrate, the enzyme activity was undoubtedly inhibited in both the Tx- and diabetic groups (Table V). The activity was completely eliminated in the diabetic group, whereas it still remained in the Tx-group under the prevailing conditions.

Discussion

Hypothyroidism and diabetes affect the fatty acid composition distribution in microsomal fractions. It has been reported that many enzymes in peripheral tissues are controlled by various hormones; Joshi and Aranda[19] reported that insulin is obligatory and thyroid hormone and other hormones are secondary to regulate enzymes in organ culture. The authors[17] demonstrated in animal experiments that the changes in serum insulin and thyroid hormone levels directly control the intracellular delta 9-desaturase. Furthermore, with this information, they speculated that such hormonal control of enzymes could be universal.

Tx and STZ-administration to rats induced hypothyroidism and diabetes. This was confirmed in Table I. Under such pathological conditions the present study was carried out to re-investigate the changes in individual fatty acid distribution in the liver microsomes (Table II). The summary of the fatty acid composition data from the present study and other investigators clearly shows, in Table IV, that the contents of C_{16} and $C_{18:0}$ specimens did not always change in the same direction by Tx; according to Faas and Carter[5] and Hoch *et al.*,[6] the magnitude of changes in $C_{16:0}$ was almost negligible, and our result in

$C_{16:0}$ was similar. However, the trends of the changes were upward in their results but downward in our result. The change in $C_{16:1}$ recorded by Faas and Carter[5] was small, but its trend was downward; whereas the result in the present study showed a clear increase. The amount of $C_{18:0}$ increased in the present study and in Hoch *et al.*[6] but it tended to be reduced in Faas and Carter.[5] In higher unsaturated C_{18}, C_{20} and C_{22} specimens, however, the Tx effect was very well matched among the three different research groups.

Regarding the effects by STZ-induced diabetes, two identical studies cited by Faas and Carter[10] and ourselves show a quite similar trend in the changes of fatty acid distribution; a clear reduction in C_{16}, $C_{18:1}$ and $C_{20:4}$ species was observed, whereas an increase in $C_{18:0}$, $C_{18:2}$, $C_{20:3}$ and $C_{22:6}$ species was seen, although the magnitude of the changes was very minor in Faas and Carter. Now, these trends in STZ-induced diabetes were compared with the profiles of the Tx-group; changes in $C_{18:2}$ and all C_{20} and $C_{22:6}$ specimens were in the same upward direction in both the treatments, except for the case of $C_{20:4}$, which was only downward. This information strongly indicates that various desaturases involved in the synthesis of polyunsaturated fatty acids are specifically affected by the two treatments, and that a different hormone deficiency affects specific sites. One of the biggest differences, but well matched among the researchers, in the fatty acid distribution due to either Tx or STZ-administration was the trend in $C_{18:1}$; the changes due to Tx were minor-upward. This was quite a contrast to the clear downward tendency of the diabetic group. Judging from the information in Tables II and III, it is likely that a *de novo* synthetic pathway ($C_{16:0} \rightarrow C_{18:1}$) is more affected by hormonal changes than the dietary-dependent synthetic pathways ($C_{18:2} \rightarrow C_{22:5}$). Furthermore, it can be said that if the minor changes are regarded within experimental deviation and the direction of the changes is negligible, the general trends in a broad spectrum of fatty acid distribution change by hypothyroidism or diabetes are essentially the same, except for the opposite direction observed in $C_{18:1}$. To obtain more details of the changes in $C_{18:1}$, the activity of delta 9-desaturase was assayed. Since the enzyme activity was completely eliminated by STZ, the increase in $C_{18:0}$ and the decrease in $C_{18:1}$ seem to be reasonable. The ambiguous effects of Tx on the $C_{18:1}$ content possibly result from a remaining activity of the desaturase (Table V). Thus, it seems likely that a small fraction of the remaining enzyme activity functions to maintain a nearly normal fatty acid distribution.

Almost two decades ago, Faas *et al.*[20] reported that thyroid hormone had no effects on chain elongation in liver microsomes *in vitro*, whereas Gnoni *et al.*[21] shortly afterwards reported a contradictory result for the thyroid hormone effect on elongation and desaturation. In contrast to both those references, the results in the present study do not reveal any "all or nothing" effect on the elongation of fatty acids. The ratios of the number of double bonds such as 16:1/16:0 in Table III reflect the activity of a corresponding desaturase, so that Tx stimulated the ratio of 16:1/16:0, but significantly reduced that of 20:4/20:3. Whereas, diabetes reduced the ratio of 18:1/18:0, which apparently indicated the inhibition of delta 9-desaturase. Diabetes also caused a rise in 18:2/18:1, indicating an increase of linoleic acid by STZ, although the possible increase of delta 6-desaturase activity was not completely ruled out.[1] The amounts of both $C_{16:0}$ and $C_{16:1}$ were reduced by diabetes, but a simultaneous increase in the ratio of C_{18}/C_{16} was observed through diabetes (Table III). In other words, the elongation of C_{16} to C_{18} is likely to be stimulated by STZ, even if the desaturation is blocked under the same conditions. Moreover, the ratio of 20:4/20:3 was heavily reduced by diabetes, but that of 22:6/22:5 was greatly stimulated. Therefore, the increase and decrease in the ratios of the number of double bonds seemingly indicate that, because of these hormones, unsaturation or desaturation of fatty acids are most likely in some specific preferential order. On the other hand, the ratios of total C_{18} content and total C_{16} content (C_{18}/C_{16}) and the like indirectly represent a corresponding elongase activity. Both Tx and STZ-induced diabetes inhibited C_{20}/C_{18}. This is in accordance with the evidence that these treatments reduced the production of arachidonic acid.[11] Interestingly, neither Tx or STZ inhibit C_{18}/C_{16} or C_{22}/C_{20}. These pieces of evidence may also support the above-mentioned idea that hormones do not exercise control everywhere at once but show a preference for a selected site.

Phospholipids and cholesterol affect the membrane fluidity and thus modify enzyme activities.[22,23] The influence of lipids on desaturases has been investigated; there was evidence that removal of a lipid portion resulted in a significant inhibition of desaturase activity,[24] while another report casts doubt on the direct interaction of lipid components with regard to enzyme activities.[25] We measured total phospholipid and total cholesterol content in microsomal fraction of Tx or STZ-induced diabetic rat livers. The Tx-effect on either total phospholipid or cholesterol content was small, but was coincidentally downward in Faas and Carter,[5] Hoch *et al.*[6] and the present study. While, on one hand, STZ-treatment clearly caused a rise in total phospholipid in liver microsomes in both Faas and Carter[10] and the present study, on the other hand, as far as the total cholesterol content is concerned, the present study showed an increase but Faas and Carter[10] reported no pertinent effects of diabetes. Huang *et al.*[11] have extensively studied phospholipid distribution in various organs from STZ-induced diabetes, but information about liver microsomes was not available; therefore, their data is not directly comparable with that of the present study. Further analyses of the effects of Tx and STZ-treatment on individual phospholipid constituents were not carried out in the present study but some figures were reported by Faas and Carter[5] for hypothyroidism, and by Huang *et al.*[11] for diabetes. According to their results, the distribution patterns of phospholipid constituents in the livers were essentially the same as those in total phospholipids found in either hypothyroidism or diabetes.

There are some items of evidence[26,27] to support the existence of graded regulation in various desaturations and elongations by treatments such as chemicals and hormones, as noted earlier. We have demonstrated[17] that delta 9-desaturase in microsomes is inversely affected by the degree of the hyperglycemic condition in the serum, and

that the enzyme activity is changed significantly by a small shift in serum hormone concentration. Therefore, the inconsistency which appeared in references to fatty acid distribution and related enzyme activities can be ascribed, in part, to a tiny variation in the physiological conditions created by the treatments.

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Different Binding Behavior to Chitin of Multiple Forms of Glucoamylases from *Aspergillus saitoi* and *Rhizopus* sp.

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Received April 10, 1991

Multiple forms of glucoamylases [EC 3.2.1.3] from *Aspergillus saitoi* and *Rhizopus* sp. were studied for their chitin binding. Two forms of *Aspergillus* enzyme, Gluc M_1 and Glu M_2, were bindable to chitin, whereas three forms of *Rhizopus* enzyme, $Gluc_1$, $Gluc_2$ and $Gluc_3$, exhibited no significant binding; of these enzyme forms, only Gluc M_1 and $Gluc_1$ are bindable to raw starch. Both Gluc M_1 and Gluc M_2, lacking the C-terminal portion of Gluc M_1, bound to chitin most favorably at pH 6.5 but with considerably different strength. The binding constants *K* of Gluc M_1 and Gluc M_2 to chitin at pH 6.5 and 4 °C were 1.8×10^6 and $0.33 \times 10^6\ \text{M}^{-1}$, respectively. Soluble starch necessitated a high concentration of 1.2% for 50% inhibition of chitin binding of Gluc M_1, as compared with 0.069% for 50% inhibition of raw starch binding (*J. Biochem.* (Tokyo), 98, 663 (1985)). Chitin-bound Gluc M_1 retained almost the same soluble starch-hydrolyzing activity as and about 3 times higher maltose-hydrolyzing and 3 times lower raw starch-digesting activities than free Gluc M_1. Thus, a chitin-binding site is not always identical with a raw starch-binding site, and in Gluc M_1 the former is located farther from the active site than the latter.

Keywords glucoamylase; chitin; binding constant; *Aspergillus saitoi*; *Rhizopus* sp.; multiple form; raw starch

Fungal glucoamylases [EC 3.2.1.3; α-D(1→4)-glucan glucohydrolase] often exist in multiple forms varying in size and hydrolytic activity on various polysaccharides, especially on raw starch.[1-7] The relationship between their structures and functions is, therefore, of interest. We have isolated three forms of glucoamylase of *Rhizopus* sp. called $Gluc_1$, $Gluc_2$ and $Gluc_3$,[8] as well as its two inactive fragments H and L,[9] and two forms of glucoamylase of *Aspergillus saitoi* termed Gluc M_1[10] and Gluc M_2[11] for comparative characterization. It has been concluded that the minor *Rhizopus* glucoamylases, $Gluc_2$ (M.W. 58600) and $Gluc_3$ (M.W. 61400), are enzyme species derived from the most abundant enzyme, $Gluc_1$ (M.W. 74000), by the action of a certain proteinase(s) with concomitant liberation of its N-terminal glycopeptides of different sizes, fragments H (M.W. 16700) and L (M.W. 14400).[8,9,12] It has been similarly considered that the minor *Aspergillus* glucoamylase, Gluc M_2 (M.W. 70000), is an enzyme species produced by proteolysis of the C-terminal, but not the N-terminal part of the major enzyme, Gluc M_1 (M.W. 90000).[10,11]

Of the multiple forms of glucoamylases, the respective largest or larger forms, $Gluc_1$ and Gluc M_1, had high raw starch-binding and raw starch-digesting activities, whereas the smaller forms, both $Gluc_2$ and $Gluc_3$ and Gluc M_2, had little raw starch-binding and much lower raw starch-digesting activities.[1,2] It was thus concluded that $Gluc_1$ and Gluc M_1 possessed separate raw starch-binding sites, different from their active sites, in the N-terminal and the C-terminal regions, respectively.[1,2] The raw starch-binding sites seemed to be located sterically near the active sites and, taken together with the results of the previous kinetic studies,[11,13] to interact not only with insoluble raw starch but also with soluble polysaccharides like soluble starch and glycogen.[1,2] It thus seems advisable that such sites be referred as polysaccharide-interacting sites rather than raw starch-binding sites.

Chitin is an insoluble polysaccharide with a β-D(1→4)-glucan structure and hence not hydrolyzable by a glucoamylase at all. However, it was unexpectedly noted that the enzymatic activity of Gluc M_1 towards raw starch was significantly inhibited by chitin, indicating binding of the enzyme to chitin. To elucidate in more detail the structure–function correlations of the multiple forms of *Aspergillus* and *Rhizopus* glucoamylases, we studied their binding behavior towards chitin, especially in relation to the behavior towards raw starch as previously reported.[1,2] The present paper deals with chitin binding of Gluc M_1 and Gluc M_2 as well as of $Gluc_1$, $Gluc_2$ and $Gluc_3$.

Materials and Methods

Chemicals Soluble starch (lot No. OCR0268) and raw starch (lot No. TOL4715) for use as substrates were purchased from Wako Pure Chemicals; the former was used after exhaustive dialysis against distilled water and the latter after successive washing with several changes each of distilled water and methanol, followed by drying over silica gel. Crustacean chitin (lot No. DWJ1274) was purchased from Wako Pure Chemicals and used after the same treatment as for raw starch. Maltose and the D-glucose oxidase reagent (Glucose C-Test Wako) were obtained from Wako Pure Chemicals. All other chemicals were of analytical reagent grade.

Preparation of Gluc M_1 and Gluc M_2 as Well as $Gluc_1$, $Gluc_2$ and $Gluc_3$ Gluc M_1 and Gluc M_2 were purified from a commercial digestive from *A. Saitoi*, Molsin (Seishin Pharm. Co., Ltd.), according to the respective methods reported previously.[10,11] $Gluc_1$, $Gluc_2$ and $Gluc_3$ were purified from a commercial digestive from *Rhizopus* sp., Gluczyme (Amano Pharm. Co., Ltd.), according to the method reported previously.[1]

Estimation of Protein Protein concentrations were determined from the absorbance at 280 nm taking A_{cm}^{280} (%) to be 14.97 and 14.18 for Gluc M_1 and Gluc M_2, respectively,[10,11] and 13.2, 13.7 and 13.4 for $Gluc_1$, $Gluc_2$ and $Gluc_3$.[8]

Determination of Glucoamylase Activity For the routine assay, glucoamylase activity was determined with soluble starch as a substrate at pH 5.0 and 37 °C according to the D-glucose oxidase method described previously.[8] One unit of glucoamylase activity was defined as the amount of enzyme liberating 1 μmol of glucose per min under the specified conditions.

The enzymatic activities with raw starch[1] and maltose[13] as substrates were determined at pH 3.5 and 5.0, respectively, as described.

Estimation of Enzyme Bound to Chitin An enzyme solution up to 100 μl was added to chitin up to 100 mg in 0.01 M acetate buffer (pH 6.5) containing 0.1 M NaCl to give a total volume of 1.1 ml. After incubation at 4 °C for 20 min with stirring, the suspension was transferred to a microfilter tube (Schleicher & Schuell, Inc.) with a filter paper and filtered by brief centrifugation at 4 °C and 3000 rpm for a few min. The resulting filtrate was used to estimate the amount of unbound enzyme, which was determined by measuring either the enzymatic activity on soluble starch or the absorbance at 230 nm instead of 280 nm because of the small amount

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of enzyme used. The amount of bound enzyme was calculated as the difference between the total and unbound enzymes, and chitin-binding activity of enzyme was expressed as the rate (%) of binding.

Estimation of Binding Constant of Enzyme to Chitin The binding constant K of an enzyme–chitin complex is expressed by Eq. 1 as follows:

$$K=\frac{B}{F(B_{max}-B)} \quad (1)$$

$$\text{thus } \frac{1}{B}=\frac{1}{B_{max}KF}+\frac{1}{B_{max}} \quad (2)$$

where B and F stand for the molar concentrations of bound and free enzymes, respectively, and B_{max} stands for the maximum molar concentration of enzyme bindable to the total amount of chitin, which is equivalent to the total molar concentration of enzyme-binding site of chitin. On the basis of Eq. 2, the value of K was estimated graphically from a plot of $1/B$ *versus* $1/F$.

Results

The bindability to chitin of the multiple forms of glucoamylases from *Rhizopus* sp. and *A. saitoi* was preliminarily explored under the same conditions as for raw starch binding (previously reported[2]) of a *Rhizopus* emzyme, Gluc$_1$. When binding of each enzyme (60—80 μg) to chitin (up to 100 mg) was examined at pH 5.0 ($I=0.1$) and 4 °C with stirring for 20 min, only *Aspergillus* enzymes, Gluc M$_1$ and Gluc M$_2$, bound to the chitin tightly or moderately whereas neither *Rhizopus* enzymes, Gluc$_1$, Gluc$_2$ nor Gluc$_3$, bound significantly. With Gluc$_1$, which is the only raw starch-bindable one of the three *Rhizopus* enzymes,[2] chitin binding was reexamined at various pH values from 3.0 to 10.0 but no detectable amounts of the enzyme were again observed to bind to chitin over the pH range tested. These results indicate that a raw starch-binding site in an enzyme does not always correspond to chitin binding, although another raw starch-bindable enzyme, Gluc M$_1$,[1] also bound to chitin. Thereafter we examined chitin binding in more detail and quantitatively using *Aspergillus* enzymes, Gluc M$_1$ and Gluc M$_2$.

To determine the optimal conditions for chitin binding of Gluc M$_1$ and Gluc M$_2$, the effect of pH on chitin binding of each enzyme was tested at 4 °C using various buffers ($I=0.1$) of from pH 2.0 to 11.0 (Fig. 1). Chitin binding of Gluc M$_1$ and Gluc M$_2$, especially of the latter, was highly pH-dependent and the most favorable binding occurred at pH 6.5 for both enzymes. The time course of chitin binding was followed with Gluc M$_1$ at pH 6.5 and 4 °C (Fig. 2). Within 3 min, about 80% of Gluc M$_1$ bound to chitin and at 10 min binding was nearly complete. As the optimal conditions for chitin binding of both Gluc M$_1$ and Gluc M$_2$, we chose 20-min incubation (to assure binding) of an enzyme with chitin at pH 6.5 (0.01 M acetate buffer–0.1 M NaCl) and 4 °C with stirring.

When a constant 1-nmol amount of Gluc M$_1$ (90 μg) or Gluc M$_2$ (70 μg) was mixed with various amounts of chitin (5—60 mg) at pH 6.5 and 4 °C, binding of both enzymes occurred hyperbolically against the amount of chitin, but the rates of binding differed considerably (Fig. 3). Gluc M$_2$ bound to chitin less tightly than Gluc M$_1$, and the amount of chitin necessary for binding of 50% of 1 nmol enzyme was about 40 mg for Gluc M$_2$ as compared with about 10 mg for Gluc M$_1$.

To estimate the binding constants K to chitin of Gluc M$_1$ and Gluc M$_2$, binding of various amounts of each enzyme to a fixed amount of chitin was measured at pH 6.5 and 4 °C. A plot of $1/B$ against $1/F$ was made according to Eq. 2; the linear slope, which is equal to $1/B_{max}K$, was calculated by least squares analysis (Fig. 4). Based on the plot, the K values for Gluc M$_1$ and Gluc M$_2$ were estimated

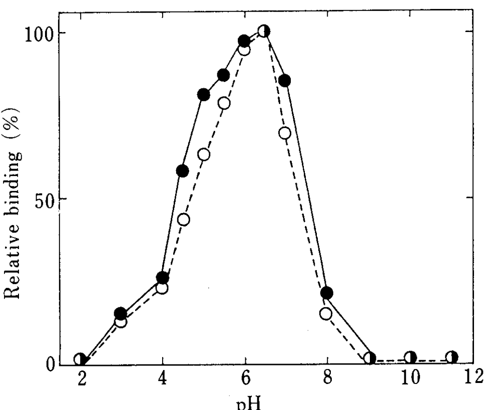

Fig. 1. Effect of pH on Chitin Binding of Gluc M$_1$ and Gluc M$_2$

Binding of Gluc M$_1$ (100 μg) and Gluc M$_2$ (78 μg) to chitin (30 mg) was measured as described in the text, except that the pH was changed from 2.0—11.0. The buffers (0.01 M, $I=0.1$) used were glycine–HCl buffer for pH 2.0—3.0, acetate buffer for pH 4.0—6.5, borax–HCl buffer for pH 7.0–9.0 and borax–NaOH buffer for pH 10.0—11.0. For each enzyme, the binding is expressed as a percentage of the maximum binding. ●, Gluc M$_1$; ○, Gluc M$_2$.

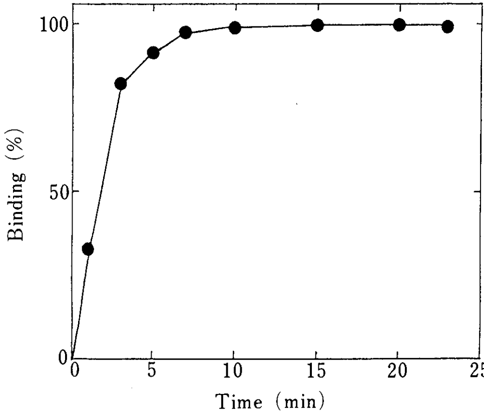

Fig. 2. Time Course of Binding of Gluc M$_1$ to Chitin

Binding of Gluc M$_1$ (100 μg) to chitin (50 mg) was measured as described in the text, except that various incubation times from 1—23 min were used.

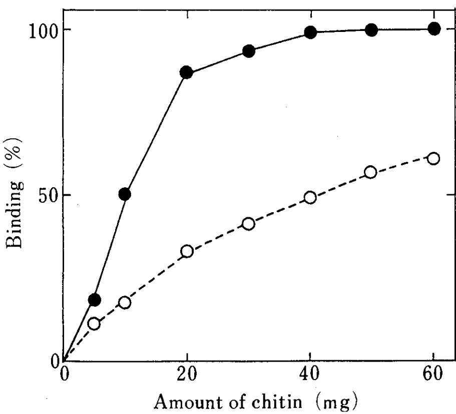

Fig. 3. Binding of Gluc M$_1$ and Gluc M$_2$ to Various Amounts of Chitin

Binding of Gluc M$_1$ (90 μg) and Gluc M$_2$ (70 μg) to various amounts of chitin from 5—60 mg was measured as described in the text. ●, Gluc M$_1$; ○, Gluc M$_2$.

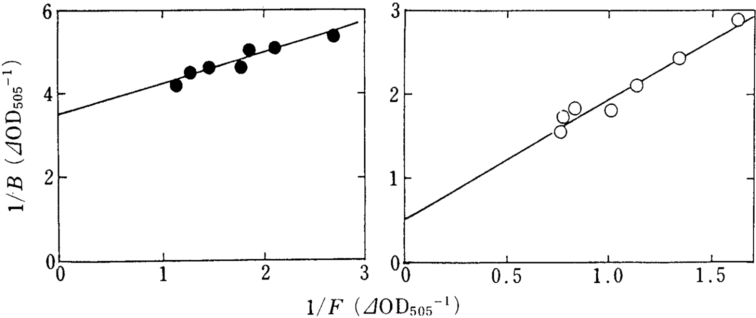

Fig. 4. Estimation of the Binding Constants of Gluc M_1 and Gluc M_2 to Chitin at pH 6.5 and 4 °C

Various amounts of Gluc M_1 (320—150 μg) and Gluc M_2 (200—90 μg) were respectively mixed with fixed amounts of chitin (10 and 50 mg) and the binding was measured as described in the text. The amounts of free (F) and bound (B) enzymes as estimated by enzymatic activity on soluble starch are expressed in terms of △ OD_{505}. ●, Gluc M_1; ○, Gluc M_2.

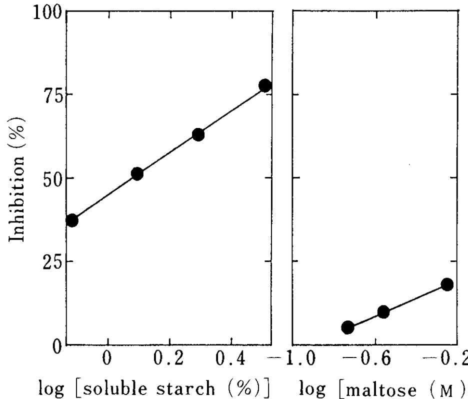

Fig. 5. Inhibition of Chitin Binding of Gluc M_1 by Soluble Starch and Maltose

Binding of Gluc M_1 (100 μg) to chitin (50 mg) was measured at pH 6.5 and 4 °C in the absence and presence of soluble starch and maltose.

to be 1.8×10^6 and $0.33 \times 10^6\,\text{M}^{-1}$, respectively.

Various saccharides which are substrates or analogs of glucoamylase showed inhibitory effects on raw starch binding of $Gluc_1$.[2] Therefore, the effects on chitin binding of Gluc M_1 of two substrates of different sizes, soluble starch and maltose, were tested (Fig. 5). Both saccharides were found to be inhibitory towards chitin binding of Gluc M_1, regardless of whether they were added before or after the formation of the complex of Gluc M_1 with chitin. However, the inhibitory effect of the saccharides on chitin binding of Gluc M_1 were much lower than those on raw starch binding of $Gluc_1$. The concentration ($I_{50\%}$) of soluble starch causing 50% inhibition was as high as 1.2% for chitin binding of Gluc M_1 as compared with 0.069% for raw starch binding of $Gluc_1$. The value of $I_{50\%}$ of maltose for chitin binding of Gluc M_1 was too high to estimate; maltose inhibited the binding by only 18% even at a concentration as high as 0.6 M.

On the other hand, chitin-bound Gluc M_1 which had been prepared by mixing 0.07 nmol of Gluc M_1 and 30 mg of chitin at pH 6.5 and 37 °C retained almost the same enzymatic activity towards soluble starch as did Gluc M_1. The identical chitin-bound Gluc M_1 had maltose-hydrolyzing activity about 3 times that of Gluc M_1 and raw starch-digesting activity only 1/3 that of Gluc M_1 (Table I). These enzyme assays were performed at 37 °C and at pH 6.5, to assure the binding of Gluc M_1 to chitin, instead of at usual pH 5.0 with soluble starch and maltose substrates and at pH 3.5 with raw starch substrate; under the assay conditions no amounts of free Gluc M_1 was practically detectable in the preparation of chitin-bound Gluc M_1.

Table I. Enzymatic Activity of Chitin-Bound Gluc M_1 towards Typical Substrates

Substrate	Enzymatic activity (%)	
	Chitin-bound Gluc M_1	Gluc M_1
Soluble starch (0.05%)	103	100
Maltose (5 mM)	321	100
Raw starch (4 mg/ml)	34	100

Enzymatic activity on each substrate was determined as described in the text, except that the buffer used was 0.01 M acetate buffer (pH 6.5) containing 0.1 M NaCl. Chitin-bound Gluc M_1 was prepared by previously mixing at 37 °C 0.07 nmol (6.3 μg) of Gluc M_1 and 30 mg of chitin in 0.5 ml of the buffer. Each substrate in 0.5 ml of the buffer was then added to give the indicated concentration and the enzymatic reaction was started. The enzymatic activity of chitin-bound Gluc M_1 is expressed as a percentage of that of Gluc M_1.

Discussion

Of the multiple forms of glucoamylases from *A. saitoi* and *Rhizopus* sp., only the respectively larger and largest ones, Gluc M_1 and $Gluc_1$, are raw starch-adsorbable and raw starch-digestible, and are considered to possess a separate raw starch-binding site(s), different from their active sites, which interacts not only with raw starch but also with other polysaccharides.[1,2] It was therefore expected that only Gluc M_1 and $Gluc_1$ which have such binding sites might also be bindable to chitin, similar to the case of glucoamylase I (GA I) of *A. awamori* var. *kawachi*. GA I, the largest of the three enzyme forms produced by the organism, which like Gluc M_1 and $Gluc_1$ is raw starch-adsorbable and raw starch-digestible,[5] was observed to be chitin-adsorbable.[14,15] Contrary to expectation, as herein described, Gluc M_1 and Gluc M_2, but not $Gluc_1$ or $Gluc_2$ and $Gluc_3$, were found to be bindable to chitin, indicating that a chitin-binding site is not always identical with a raw starch-binding site.

Chitin binding of Gluc M_1 and Gluc M_2 occurred more pH-dependently, with the maximum binding at pH 6.5, than raw starch binding of either Gluc M_1 or $Gluc_1$, the maximum binding of which occurred at pH 3.0 and pH 4.5—5.5, respectively.[1,2] On the other hand, it was claimed that chitin binding of GA I occurred quite pH-

independently, although raw starch binding of GA I was pH-dependent.[15] This pH-independency of GA I might merely have reflected the phenomenon that GA I bound to chitin so tightly that under the experimental conditions complete binding of GA I to chitin was consistently obtained over the pH range tested. Anyway, the differences in optimal pH for binding to chitin or raw starch of the enzymes seem to result from the different structures of the respective binding sites.

Gluc M_1 tightly bound to chitin with a binding constant K of $1.8 \times 10^6\ \text{M}^{-1}$ at pH 6.5 and 4 °C; this is the first reported K value of a chitin–glucoamylase complex. The K value was about 10 times larger than those of the raw starch complexes with Gluc M_1 ($1.6 \times 10^5\ \text{M}^{-1}$)[1] and Gluc_1 ($1.2 \times 10^5\ \text{M}^{-1}$),[2] indicating more tight binding of Gluc M_1 to chitin than to raw starch. This seems to be true also with GA I, considering that GA I completely bound to chitin throughout the pH range tested while it only partially bound to raw starch outside the optimal pH range.[15] Gluc M_2, which lacks the C-terminal portion (about 20000 dalton) of Gluc M_1 and fails to bind to raw starch, still retained the ability of Gluc M_1 to bind to chitin, although only partially. The K value ($0.33 \times 10^6\ \text{M}^{-1}$) of the chitin complex with Gluc M_2 became about 1/6 times that of Gluc M_1. This, together with the previous results,[1,10,11] implies that the chitin binding site of Gluc M_1 is located farther inside the C-terminal region than the raw starch binding site, and that the former site, some part of which is also in the C-terminal region of Gluc M_2, is almost, if not completely, separated from the latter site.

Chitin-bound Gluc M_1 retained almost the same soluble starch-hydrolyzing activity as and 3 times lower raw starch-digesting activity than free Gluc M_1.Chitin-bound GA I also retained most of the original activity of free GA I towards gelatinized starch and glycogen, but it lost completely the original raw starch-binding and raw starch-digesting activities.[14] These results indicate that the chitin binding sites of both Gluc M_1 and GA I are located separate from their active sites and that the chitin binding sites are located near their raw starch binding sites. Thus it is considered that the chitin binding site of Gluc M_1, which was found in the present work to reside farther inside the C-terminal region than the raw starch binding site, is folded sterically farther from the active site than the raw starch binding site. This is not incompatible with the present finding that soluble starch necessitated a high concentration of 1.2% for 50% inhibition of chitin binding of Gluc M_1, compared with a low concentration of 0.069% for 50% inhibition of raw starch binding of Gluc_1,[2] although the enzymes are not identical. Chitin-bound Gluc M_1 also showed about 3 times higher maltose-hydrolyzing activity than Gluc M_1. Binding of Gluc M_1 to chitin may result in a certain steric change in the active site, consisting of about 7 substites,[16] thereby increasing its productive binding with maltose as a substrate.

Chitin is the main component of fungal cell wall. It is not a substrate of glucoamylase and the reason it binds tightly with Gluc M_1, Gluc M_2 and GA I is not known. Chitin may be an effective carrier of such enzymes without the aid of a cross linking agent.

Acknowledgment The authors are greatly indebted to Dr. F. Yoshida of Kikkoman Shoyu & Seishin Pharm. Co. and Amano Pharm. Co. for the kind supply of the enzyme sources Molsin and Gluczyme, respectively.

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Preparation and Evaluation of Oral Dosage Form Using Acylglycerols. II.[1] Effect of Food Ingestion on Dissolution and Absorption of Aspirin from the Granules Prepared by Acylglycerols in Human Subjects[2]

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The dissolution behavior of the aspirin enteric granule prepared using acylglycerols, glyceryl monostearate (GMS) and glyceryl trilaurate (GTL), was investigated *in vitro* and in human subjects in a fasting or non-fasting state. Aspirin was slowly released from the granule *in vitro* at pH 1.2. No acceleration of the aspirin dissolution rate in the medium without lipase and cholic acid was observed when the pH level of the medium increased to a neutral region (pH 6.4). However, the dissolution of aspirin was significantly increased by increasing the concentrations of lipase and cholic acid in the medium. Lipase appears to play an essential role in the dissolution process of aspirin granules. In human subjects, the average levels of the cumulative amount of total salicylate excreted in a urine–time curve, and the mean residence time (*MRT*) obtained after oral administration of a granule in the fasting state were markedly delayed in comparison with the results observed using an aqueous solution and a crystalline form of aspirin. In comparing the fasting condition with the non-fasting condition (after food ingestion), no significant difference was recognized in the total amount of salicylate excreted in urine to an infinite time ($A_e(\infty)$), whether the *MRT* was obtained by granule, crystalline form or aqueous solution. It can be concluded that aspirin granule prepared by GMS and GTL has a property of pancreatic lipase-sensitive dissolution, and its bioavailability is unaffected by food intake.

Keywords aspirin enteric granule; mean residence time; food ingestion effect; acylglycerol; glyceryl monostearate; glyceryl trilaurate; human subject; gastrointestinal absorption

Introduction

An enteric preparation, which has a pH-independent dissolution property, may offer promising drug release in patients with low gastric acidity, *e.g.* anacidity and achylia. Recently, we reported that pH-independent enteric granules containing aspirin could be prepared by combining aspirin and glyceryl monostearate (GMS) mass coated with melted glyceryl trilaurate (GTL) using a centrifugal rotating mixer.[1] These acylglycerols, edible and safe pharmaceutical ingredients, are digested by lipase and bile salts in intestinal juice. Consequently, acylglycerols provide a useful means of preparing a pH-independent (pancreatic lipase-sensitive) enteric granule containing aspirin. In a dissolution test *in vitro*, aspirin could not be released (dissolution percentage, below 10% within 2 h) from the prepared granule in a pH 1.2 aqueous solution, whereas the dissolution percentage of aspirin was increased (about 80% at 2 h) in a pH 6.4 phosphate buffer solution (PBS) containing lipase and cholic acid.

In the present investigation, to better understand the dissolution behavior of aspirin granules prepared using acylglycerols, absorption of aspirin from the granules was evaluated in healthy human subjects after oral administration. The aim of this study was to compare absorption from the aspirin granule with that from a crystal form in an aqueous solution, under a fasting condition as well as when taken with a standardized meal (food ingestion).

Experimental

Materials Aspirin (JP XI) was obtained from Iwaki Seiyaku (Tokyo, Japan). Acylglycerol: GMS (Riken Vitamin, Tokyo, Japan) was used after the removal of free fatty acids and glycerol according to the method described in our previous report,[1] and GTL (Tokyo Kasei Kogyo, Tokyo, Japan) was used. Lipase (triacylglycerol lipase, EC 3.1.1.3) and cholic acid (sodium salt) were purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals obtained were of commercial analytical grades.

Preparation of Aspirin Granules Aspirin granules using GMS and GTL were prepared by the two-step dry mixing method, as described in our previous paper.[1] Aspirin powder (80—100 mesh) was aggregated by melted GMS; the aspirin-GMS mass was formed. Subsequently, the aspirin granule (5.5—12 mesh) coated with melted GTL was prepared using crushed aspirin-GMS masses of 12—42 mesh. After GTL coating, free GTL which did not adhere to the surface of crushed aspirin-GMS mass was removed by sieving. The content-ratio of aspirin (50%), GMS (25%) and GTL (25%) in granules was determined by the spectrophotometric method described in our previous report.[1]

***In Vitro* Dissolution Test** The dissolution rates of aspirin from granules were determined in a manner similar to the paddle method described in JP XI, using a dissolution test apparatus (model NTS-VS, Toyama Sangyo, Osaka, Japan). A sample of 200 mg calculated as aspirin was tested. Dissolution media were 500 ml of the following aqueous solution: pH 1.2 solution (the first fluid for disintegration test, JP XI), pH 6.4 PBS[3] and PBS containing lipase and cholic acid, at a constant temperature (37±0.1 °C). The dissolution medium was stirred at 100 rpm with a paddle stirring element. At predetermined time intervals, an aliquot of 4 ml of the dissolution medium was taken, and the medium was replenished with the same volume of each solution. Aspirin was not hydrolyzed by lipase and cholic acid in the dissolution medium during the experimental period.

The dispersion was immediately filtered through a 0.2 μm cellulose nitrate membrane filter (Dismic-25, Toyoroshi, Tokyo, Japan) to remove the particles. The concentration of salicylic acid was assayed by measuring the absorbance (300 nm) on an ultraviolet (UV)-visible spectrophotometer (model UV-240, Simadzu Seisakusho, Kyoto, Japan) after hydrolysis of aspirin with 1 N NaOH. The absorbance of salicylic acid was determined by subtracting the absorbance of lipase and cholic acid in the dissolution medium without salicylic acid (blank test) from observed absorbance in the dissolution medium.

***In Vivo* Studies** Male volunteers (21 to 25 years old, weighing 60 to 70 kg), from whom informed consent was obtained, participated in this study. All subjects were in good health and none were taking any other medication. After overnight fasting, the subjects received single oral administration of 500 mg of aspirin crystal (12—42 mesh) or granule containing 500 mg of aspirin together with 200 ml of water in the fasting state. Participants in the control group received 200 ml of aqueous solution containing 500 mg of aspirin instead of the crystalline form or granules. The subjects in the non-fasting state received the same preparations as those subjects in the fasting state 30 min after a

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standard breakfast. The standard breakfast[4] consisted of 100 g of bread, 20 g of butter, a boiled egg, 35 g of cucumber and 200 ml of milk. All subjects abstained from any other food or liquid until 6 h after dosing, and then took food and drink *ad libitum*. Each dosage was separated by at least a week.

Urine samples were collected immediately before drug administration and at predetermined time intervals following administration. After the urine volume was measured, urine samples were kept frozen until analysis. With respect to the observation of bioavailability of aspirin dosage forms such as powder, tablet and enteric-coated preparations in human subjects, the determination of total salicylate in urine has been reported.[5-7] In this study, therefore, total salicylate excreted in urine was determined by the colorimetric method.[8,9]

Data Analysis The statistical moments for the urinary excretion rate–time curve are defined as the following equations[10]:

$$A_e(\infty)=\int_0^\infty (dA_e/dt)dt \quad (1)$$

$$MRT=\int_0^\infty t(dA_e/dt)dt \Big/ \int_0^\infty (dA_e/dt)dt \quad (2)$$

dA_e/dt is the urinary excretion rate, $A_e(\infty)$ is total amount of drug excreted in urine to an infinite time, and MRT is the mean residence time. These symbols are in accord with the proposal of Rowland and Tucker.[11] $A_e(\infty)$ and MRT were calculated by linear trapezoidal integration with extrapolation of the time course curve to infinite time according to a monoexponential equation.

The one-way ANOVA and Dunnett's tests were employed for statistical evaluation. Differences were considered to be significant at the $p<0.05$ level for all experiments.

Results and Discussion

Dissolution of Aspirin from the Granules Prepared by Acylglycerols *in Vitro* Figure 1 illustrates the mean dissolution percentage–time curves of aspirin from granules at pH 1.2 (shown as unfilled triangles) and 6.4 (shown as filled triangles). Aspirin crystal rapidly dissolved in an aqueous solution at pH 1.2 (dissolution percentage: 100% within 30 min). However, aspirin was slowly released from granules in the dissolution medium at pH 1.2. Consequently, a very low dissolution percentage (less than 10%) was obtained at 2 h. When the pH value was increased to a neutral region (pH 6.4), the dissolution percentage did not increase remarkably (approximately 20% or below at 2 h) in the dissolution medium without lipase and cholic acid (represented by the broken line with filled triangles). These results show that the dissolution of aspirin from granules is not influenced by changes in the pH level.

When lipase and cholic acid were added to the dissolution medium at pH 6.4 (represented by the straight line with filled triangles), the dissolution of aspirin from granules increased significantly. The dissolution percentage (*ca.* 75%) at 2 h was approximately 4 times higher than at 2 h in the medium without lipase and cholic acid. Aspirin was released more efficiently from granules in the dissolution medium containing lipase and cholic acid than in the medium without both materials. However it is difficult to define the optimal concentration of pancreatic lipase and cholic acid in the *in vitro* dissolution test, since the secretion of both materials is changed by various physiological conditions, for instance, following the consumption of food. The maximal effect of lipase and gall powder on the lipid digestion of the triglyceride spheres containing sulfamethizole at a concentration of 0.4% has been reported.[12] In our preliminary observation, the effective concentrations of lipase and cholic acid on the digestion of prepared aspirin granules were obtained at 0.6—1.0% and 0.1—0.2%, respectively. Concerning the effectiveness of lipase and cholic acid on dissolution, lipase appears to play an essential role in the action. It was confirmed that the aspirin is dissolved from granules prepared using GMS and GTL by lipase.

Dissolution Behavior and Absorption of Aspirin from Granules *in Vivo* The aspirin granules prepared using

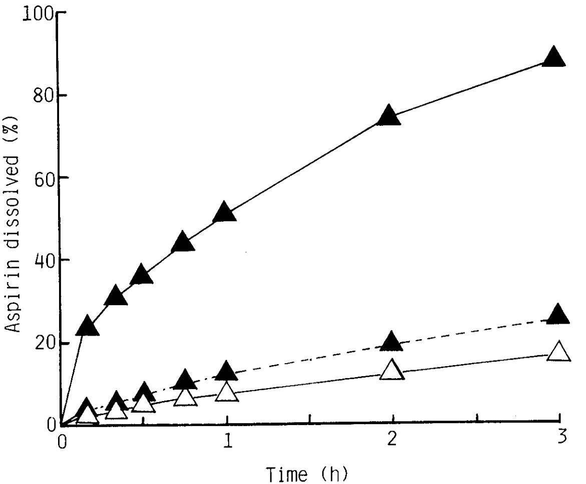

Fig. 1. Dissolution Curves of Aspirin from Prepared Granules in the Dissolution Medium at pH 1.2 and 6.4 with or without Lipase and Cholic Acid

Dissolution medium without lipase and cholic acid: —△—, pH 1.2; ---▲---, pH 6.4. Dissolution medium with lipase (0.6%) and cholic acid (0.1%) at pH 6.4: —▲—. Points are expressed as the mean of three experiments.

$$\text{Dissolution (\%)}=\frac{\text{amount of aspirin dissolved in medium}}{\text{amount of aspirin added}}\times 100 .$$

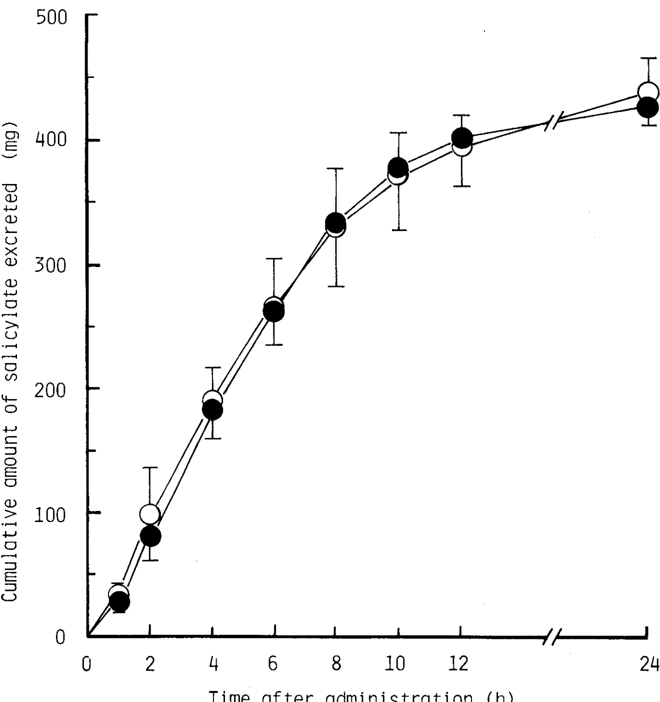

Fig. 2. Average Cumulative Amount of Urinary Excretion–Time Curves of Total Salicylate after Oral Administration of Aspirin Aqueous Solution in Human Subjects

Key: —○—, fasting state; —●—, non-fasting state. Each value represents the mean of six experiments and the vertical bar indicates S.D.

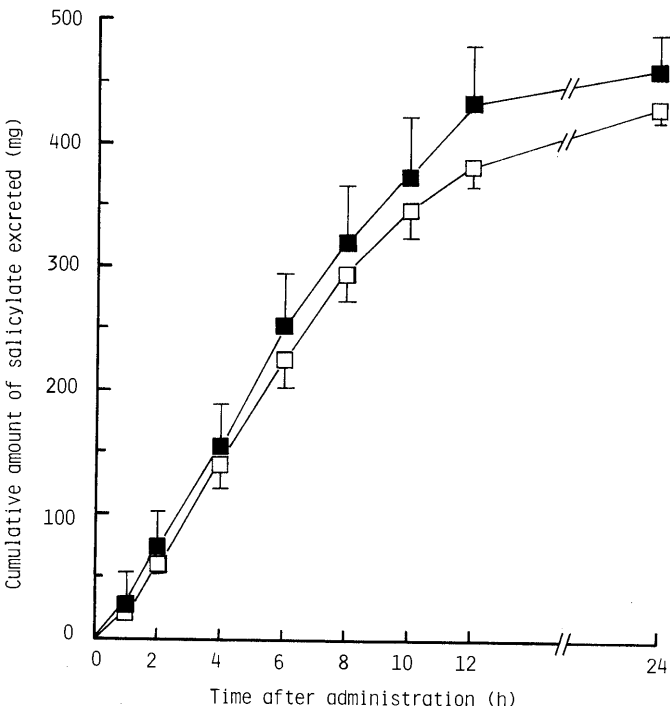

Fig. 3. Average Cumulative Amount of Urinary Excretion–Time Curves of Total Salicylate after Oral Administration of Aspirin Crystal in Human Subjects

Key: —□—, fasting state; —■—, non-fasting state. Each value represents the mean of six experiments and the vertical bar indicates S.D.

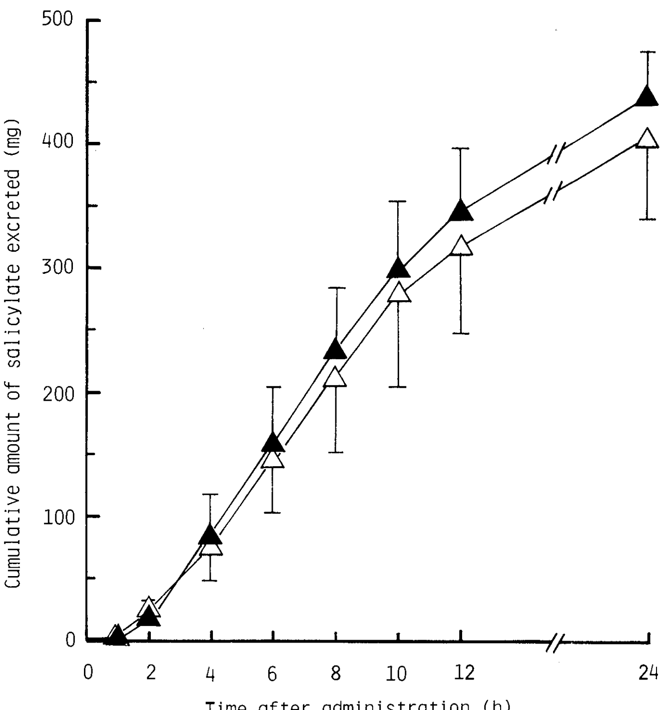

Fig. 4. Average Cumulative Amount of Urinary Excretion–Time Curves of Total Salicylate after Oral Administration of Prepared Aspirin Granule in Human Subjects

Key: —△—, fasting state; —▲—, non-fasting state. Each value represents the mean of six experiments and the vertical bar indicates S.D.

GMS and GTL are digested by pancreatic lipase. Intake of food may influence the dissolution of aspirin from these granules. To clarify the effect of food ingestion on the dissolution behavior and the gastrointestinal absorption of aspirin from granules, the cumulative amount of total salicylate excreted in urine after oral administration of aspirin granules was evaluated in human subjects in the fasting state or non-fasting state (after meals). Aspirin in an aqueous solution or in crystalline form was tested as the reference preparation.

TABLE I. Total Amount of Salicylate Excreted in Urine to Infinite Time ($A_e(\infty)$) and *MRT* Following Oral Administration of Aspirin Preparation in Fasting or Non-fasting State Human Subjects

Preparation	$A_e(\infty)$ (mg)		*MRT* (h)	
	Fasting	Non-fasting	Fasting	Non-fasting
Aq. solution	449 ± 44	428 ± 24	6.5 ± 2.3	5.6 ± 1.2
Crystal	437 ± 11	466 ± 27	7.2 ± 1.0	6.7 ± 1.5
Granule	431 ± 64	471 ± 50	$10.2 \pm 2.2^{a)}$	$10.4 \pm 3.3^{b,c)}$

Each value represents the mean ± S.D. of six experiments. Statistically significant differences: *a*) $p<0.05$ in granule *vs.* aq. solution; *b*) $p<0.01$ in granule *vs.* aq. solution; *c*) $p<0.05$ in granule *vs.* crystal.

The mean cumulative amount of urinary excretion–time curves after oral administration of aspirin solution, powder and granule in a fasting state (shown as unfilled symbols) and non-fasting state (shown as filled symbols) are illustrated in Figs. 2, 3 and 4, respectively. $A_e(\infty)$ and *MRT* obtained after administration of aspirin preparations are summarized in Table I. In the case of aqueous solution, aspirin was rapidly absorbed from the gastrointestinal tract in the non-fasting condition as well as the fasting condition. The rapid absorption of aspirin observed in our study is in general agreement with the results of Levy *et al.*[13] No significant difference in the cumulative amount of urinary excretion–time curves between the two conditions was found (Fig. 2). The elimination rate constants ($0.230 \pm 0.083\,h^{-1}$ in the fasting state: $0.306 \pm 0.120\,h^{-1}$ in the non-fasting state) of total salicylate obtained by the calculation method of Levy [14] were not significant. These results suggest that gastrointestinal absorption and elimination of aspirin from an aqueous solution is not influenced by foods.

When aspirin was administered in a crystalline form, aspirin was also efficiently absorbed. The average levels of the cumulative amount of urinary excretion from a crystalline form (Fig. 3) were not lower than those from an aqueous solution (Fig. 2), and no significant difference was recognized in $A_e(\infty)$ and *MRT* between both forms in the fasting condition (Table I). Therefore, aspirin could dissolve rapidly in gastrointestinal fluid. After food ingestion, mean values of $A_e(\infty)$ (466 ± 27 mg) and *MRT* (6.7 ± 1.5 h) were similar to those ($A_e(\infty)$, 437 ± 11 mg: *MRT*, 7.2 ± 1.0 h) obtained in the fasting condition. It can therefore be presumed that foods do not induce a change of aspirin dissolution from the crystalline form in gastrointestinal fluid.

It is generally accepted that the gastric emptying rate of pharmaceutical preparations is influenced by coadministered drugs or food intake.[15] The effect of food ingestion on the gastric emptying rate of aspirin in aqueous solution or crystalline form may be ignored in evaluating the bioavailability of aspirin in human subjects since similar results of $A_e(\infty)$ and *MRT* were obtained between the fasting and non-fasting states.

The aspirin granule significantly delayed the urinary excretion until 12 h after administration (Fig. 4) and increased MRT values in the fasting state (10.2 ± 2.2 h) as well as in the non-fasting state (10.4 ± 3.3 h). These results suggest that aspirin in granules dissolved slowly in the gastrointestinal tract. However, no significant difference of $A_e(\infty)$ values from granules between the two conditions was found.

Considerable attention has been paid to the effect of foods on the absorption characteristics of aspirin from various oral preparations such as conventional tablets,[4,16] enteric-coated tables[17,18] and granules,[17,18] *etc.*, in volunteers. Furthermore, the influence of food ingestion on the gastric emptying rate of an oral dosage form of aspirin has been studied in humans.[19,20] Taken with food, the enteric-coated tablets gave a much lower absorption than the enteric-coated granules which were not influenced by the intake of food.[18] Moreover, the dosage form with a larger size was found to be more slowly emptied from stomach,[19] and the gastric empyting rate of aspirin granules (diameter, 1 mm) was less affected by food than that of tablets (diameter, 4—8 mm).[20] These findings suggest that aspirin granules permit more reproducible absorption than tablets.

The observation made in this study is that the aspirin granules prepared using GMS and GTL show delayed absorption compared with the aqueous solution or crystalline form. However, there was no detectable influence of food on aspirin absorption from granules. The granules were obtained by sieving the product through 5.5—12 mesh (diameter, 3.35—1.40 mm). Probably, the gastric emptying rate of these granules is not affected by food ingestion.

Generally, intake of food enhances the secretion of lipase and bile salts.[21] However, it is impossible to determine the exact concentrations of pancreatic lipase and cholic acid in intestinal juice during the fasting or non-fasting condition. Although the difference of concentration of these materials between two conditions may influence the dissolution of aspirin from the prepared granules in the small intestine, aspirin can dissolve from granules at a low concentration (only less than 1%) of lipase. Thus, this may be one of the reason why significant differences of $A_e(\infty)$ and MRT were not observed between the fasting and non-fasting states.

In conclusion, the aspirin granules prepared by GMS and GTL have a property of pancreatic lipase-sensitive dissolution, and its bioavailability is unaffected by food intake. This aspirin granule is a useful dosage form of a delayed-release preparation having a pH-independent dissolution.

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Study on the Cupric Phenanthroline-Induced β-Glucuronidase Release in Saponin-Permeabilized Polymorphonuclear Leukocytes

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Saponin-permeabilized polymorphonuclear leukocytes (PMNs) released β-glucuronidase, a lysosomal enzyme, dose-dependently in response to cupric phenanthroline (CuPh), a mild oxidant, which catalyzes the formation of disulfide bridges. The β-glucuronidase release induced by CuPh was inhibited by ethylene glycol bis(β-aminoethylether)-*N,N,*-*N′,N′*-tetraacetic acid (EGTA). Both dithiothreitol (DTT) and *N*-(6-aminohexyl)-5-chloro-naphthalene sulfonamide (W-7) also inhibited the β-glucuronidase release induced by CuPh. CuPh elicited a decrease in protein-bound free sulfhydryls simultaneously, and this decrease was not restored by EGTA treatment. CuPh inhibited Ca^{2+} uptake into Ca^{2+} store sites, and promoted a Ca^{2+} efflux from Ca^{2+} store sites. It also inhibited Ca^{2+}-adenosine triphosphatase (ATPase) activity in permeable PMNs. DTT, a sulfhydryl reducing agent, suppressed both the β-glucuronidase release and the Ca^{2+} uptake in CuPh-treated permeable PMNs. On the other hand, chloromercuriphenylsulfonic acid (CMPS), a sulfhydryl modifier, decreased the amount of free sulfhydryls in protein and released β-glucuronidase in permeable PMNs dose-dependently, but EGTA did not inhibit either reaction. Neither CuPh nor CMPS released β-glucuronidase from intact PMNs.

These results indicate that both CuPh and CMPS act on intra-PMN target molecules to exert their influence, but the involved mechanisms are different in nature. Alteration in calcium movement is responsible for the β-glucuronidase release in the CuPh-treated permeable PMNs.

Keywords polymorphonuclear leukocyte; permeabilization; cupric phenanthroline; calcium mobilization; degranulation; beta-glucuronidase release; sulfhydryl reagent; disulfhyde linkage; saponin

Introduction

Intracellular sulfhydryl groups are essential to biological responses in various cellular systems. Oxidation of non-glutathione sulfhydryls was reported to be one of the biochemical changes which contributes to C5a-induced aggregation in polymorphonuclear leukocytes (PMNs).[1] It was suggested that changes in sulfhydryl status by reactive aldehydes modulate the activity of the plasma membrane reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase responsible for superoxide anion production in stimulated PMNs and pulmonary alveolar macrophages.[2] Rice and Barnea reported that copper-mediated oxidation of thiols regulated the release of luteinizing hormone releasing hormone from isolated hypothalamic granules in adult male rats. They proposed that copper, bound to an intracellular chelator (protein, peptide, or amino acid), oxidizes thiols of the granule, leading to a change in granule-membrane permeability and hence to the hormone release.[3]

Intracellular free Ca^{2+} has been implicated as a second messenger in stimulus-response coupling in various cells. Using permeable cells, extracellularly added Ca^{2+} stimulated mast cells,[4] adrenal medullary chromaffin cells,[5] GH_3 pituitary cells,[6] PC12 cells,[7] isolated gastric glands,[8] platelets[9] and neutrophiles.[10] Moreover, in permeable platelets, sulfhydryl-reacting reagents decreased the protein-bound free sulfhydryls, changed Ca^{2+} movement, and elicited a release reaction. The report suggested that protein-bound free sulfhydryls play an important role in intracellular Ca^{2+} metabolism and hence various biochemical and biological functions of platelets.[11] However, the significance and role of the exchange between free sulfhydryls and disulfide bonds for lysosomal enzyme release in PMNs, especially in relation to intracellular Ca^{2+} movement, are not yet clearly understood.

In this report, PMNs were permeabilized with a cholesterol-complexing agent—saponin—and thus, permeabilized PMNs responded to *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), a physiological stimulator for PMNs. These experiments revealed that permeabilized PMNs are similar to intact PMNs in regard to their response to physiological stimulators. These permeable PMNs secreted a lysosomal enzyme, β-glucuronidase, in response to cupric phenanthroline (CuPh) or chloromercuriphenylsulfonic acid (CMPS). Using these permeable PMNs, the effect of CuPh, a sulfhydryl oxidizer,[12] and CMPS, a sulfhydryl-modifying agent, on protein-bound sulfhydryl content and Ca^{2+} movement was examined to evaluate the relation among intracellular sulfhydryl modification, Ca^{2+} movement, and β-glucuronidase release.

Materials and Methods

Materials NADH (grade III, from yeast), W-7, fMLP, phospho(enol)-pyruvate trisodium salt hydrate, NAD and oligomycin were obtained from Sigma (St. Louis, Mo., U.S.A.); $^{45}CaCl_2$ (24.6 mCi/mg calcium) was from Du Pont/New England Nuclear (NEZ-013, Boston, U.S.A.); Adenosine triphosphate (ATP) was from Fluka AG (Buches, Switzerland); pyruvate kinase (type III, 213 units/mg) and lactate dehydrogenase (LDH, type II, 2930 units/ml) were from Toyobo Co., Ltd. (Osaka, Japan); *p*-chloromercuriphenylsulfonic acid monosodium salt, *p*-nitrophenyl-β-D-glucuronide and ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) were from Nakarai Chemical Ltd. (Kyoto, Japan); 1,10-*ortho*-phenanthroline, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 1,4-dithiothreitol (DTT) and *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and membrane filters (type TM-2, cellulose nitrate, 0.45 μm) were from Toyo Roshi Co. Ltd. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Preparation and Permeabilization of PMNs Male guinea pigs of the Hartley strain were intraperitoneally injected with 26 ml of 2.9% thioglycollate 15 h before the harvest of peritoneal exudate cells. The cells were harvested from the peritoneal cavity using modified Hanks' balanced salt solution (20 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, 0.5 U/ml heparin). The collected cells were centrifuged (110 × *g*, at 4 °C, for 10 min) and washed with the same buffer. Afterwards leukocyte cell density was adjusted to

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2.0×10^7/ml in a reaction buffer (30 mM HEPES, pH 7.0, 100 mM KCl, 20 mM NaCl), and saponin (Wako Pure Chemical) was added to the culture to make a 105 μg/ml concentration. The cell suspension was incubated for 10 min at 37 °C, washed with the reaction buffer by centrifugation ($150 \times g$, at 4 °C, for 5 min), and resuspended in the reaction buffer at appropriate cell density. Thus permeabilized PMNs were incubated with various reagents such as CuPh. In the case of CuPh, a 3/1 molar ratio of *o*-phenanthroline/$CuSO_4$ was used. Hereafter, CuPh concentration denotes the *o*-phenanthroline concentration.

β-Glucuronidase and LDH Measurement β-Glucuronidase activity was assayed with *p*-nitrophenyl-β-D-glucuronide (Nakarai Chemical Ltd.) as a substrate. The assay mixture contained: 50 mM sodium acetate buffer (pH 5.0), 0.1% (w/v) Triton X-100, 1 mM *p*-nitrophenyl-β-D-glucuronide and culture supernatant. Permeabilized PMNs (0.8×10^7/ml: 400 μl) were placed in conical centrifugation tubes and incubated for 15 min at 37 °C in the presence of various reagents, and the reaction was terminated by centrifugation ($6000 \times g$, at room temperature, for 1 min). Aliquots of supernatants (50 μl) were obtained and submitted to the measurement of β-glucuronidase and LDH activities. β-Glucuronidase activity was determined by measuring the *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucuronide.[13] LDH [EC 1.1.1.27], which is a cytoplasmic marker enzyme,[14] was quantified by the method of Kornberg.[15] Total β-glucuronidase and LDH were determined by solubilizing PMNs with 0.5% Triton X-100, and released substances were expressed as a percentage of the total amounts.

Ca^{2+} Movements Ca^{2+} uptake was measured by using $^{45}Ca^{2+}$ and the Millipore filtration technique. Permeabilized PMNs (0.62×10^7/ml: 260 μl) were suspended in the reaction buffer containing 1.5 mM Mg/ATP, 77 μM $CaCl_2$ and 1.0 μCi $^{45}Ca^{2+}$, and incubated at 25 °C for 30 min. The cell suspension was passed through a membrane filter (0.45 μm). The filter was washed twice with the same medium without $^{45}Ca^{2+}$ of 1.0 ml, dried and counted for radioactivity with a liquid scintillation counter (LSC-900, Aloka). The ratio of Ca^{2+} uptake was calculated dividing the value by that of the control experiment, which contained saline in place of CuPh.

Ca^{2+} efflux was determined by the method described by Chiesi.[16] After $^{45}Ca^{2+}$ had loaded into the permeable PMNs (1.0×10^8, 25 °C, 3 h) in a buffer containing 1.7 mM Mg/ATP, 87 μM $CaCl_2$ and 10 μCi/ml of $^{45}Ca^{2+}$ (25 mCi/mg), a passive efflux experiment of Ca^{2+} was started by diluting Ca^{2+}-loaded PMNs 27 times with the reaction buffer containing 0.25 mM EGTA and various reagents. The PMNs were incubated for 10 min, then the mixture was filtered (within 10 s). The filter was washed and dried, and its radioactive count was determined. The Ca^{2+} efflux was expressed by the ratio of remaining $^{45}Ca^{2+}$ count in permeable PMNs of a test sample-treated to that in the control culture.

Quantification of Sulfhydryl Groups in Proteins The quantification of PMN sulfhydryl groups in proteins was carried out by the method of Yamada *et al.*[17] The precipitate of PMNs was suspended in 200 μl of distilled water after being treated with various reagents (CuPh, EGTA *etc.*). The suspension was mixed with a precipitating solution containing 1.67% *m*-phosphoric acid, 0.02% ethylenediaminetetraacetic acid disodium salt, and 30% NaCl. It was then allowed to stand for about 2 h at room temperature, followed by centrifugation at $8500 \times g$ for 1 min. The precipitate was used for the quantification of protein-bound sulfhydryl groups, and the denatured protein sediment was neutralized by the addition of 0.1 ml of 0.3 M Na_2HPO_4 and solubilized by adding 1 ml of 1% sodium dodecyl sulfate (SDS). A 0.2 ml solution was saved for protein quantification. The rest of the solution was mixed with a 10 mM DTNB solution of 81.8 μl. After incubation for 30 min at room temperature, optical density was measured at 412 nm. The content of sulfhydryl groups was calculated on the basis of a molar extinction coefficient of 1.16×10^4. Protein content was determined according to Lowry *et al.*[18] using bovine serum albumin as a standard. The amount of free sulfhydryls in proteins was expressed as pmoles per μg protein.

Ca^{2+}-Dependent ATPase Activity ATPase activity was essentially measured as described by Neet and Green,[19] using NADH, pyruvate kinase and LDH. Typically, permeable PMN suspension (1.0 ml, 0.8×10^7/ml) was incubated with CuPh at 37 °C for 10 min. CuPh was removed by centrifugation ($150 \times g$ for 5 min at 25 °C). PMN pellet was resuspended to the same cell density in the reaction buffer. An aliquot (350 μl) of cell suspension was diluted to 1.5 ml with a solution which contained 0.55 mM phospho(enol)pyruvate, 0.2 mM NADH, 6.5 mM $MgCl_2$, 1.3 mM ATP, 1.3 units/ml LDH, 1.3 units/ml pyruvate kinase, and 1.3 mM EGTA and 2.6 mM $CaCl_2$. Changes of absorbance at 340 nm (NADH) were recorded using a double-beam spectrophotometer (model 200-20, Hitachi Ltd., Tokyo) at room temperature. Basal ATPase activity was measured following the addition of 1.3 mM ATP. Thereafter, 2.6 mM $CaCl_2$ was added, and the Ca^{2+}-requiring ATPase activity was detected by a decrease in light absorption at 340 nm. Ca^{2+}-dependent ATPase activity was calculated from the difference between the initial velocities of the Ca^{2+}-requiring ATPase and basal ATPase, and it is expressed as nmols of ATP converted per min per 10^7 PMNs.

Results

Permeabilization of Guinea Pig PMNs Guinea pig PMNs were treated with various concentrations of saponin as described in Materials and Methods, and leakages of LDH and β-glucuronidase were assessed simultaneously in order to establish optimum conditions for permeabilization. The leakage of β-glucuronidase was less than that of LDH (a cytoplasmic marker enzyme) in every saponin concentration examined. The percent releases of LDH and β-glucuronidase were 50 and 5, respectively, when PMNs were treated with 105 μg/ml of saponin for 10 min. This result indicates that the plasma membrane became permeable, while the lysosomal granule membrane remained intact under these experimental conditions. The permeabilized PMNs released β-glucuronidase in response to fMLP, as in the case of intact PMNs. This indicates that there is minimal disturbance to the topography of the surface membrane receptor for fMLP and of its function for granule secretion.

Effect of CuPh on β-Glucuronidase Release from Permeable PMNs CuPh (an -S–S- cross-linker) released β-glucuronidase from saponin-permeabilized PMNs dose dependently up to 600 μM (Fig. 1). Under these experimental conditions, constant leakage of LDH (about 50%) was observed, and it was dependent on the saponin concentration but not on the concentration of CuPh (up to 1 mM). No release of β-glucuronidase occurred from intact PMNs (not treated with saponin) by CuPh up to the concentration of 600 μM. EGTA inhibited the release of β-glucuronidase induced by CuPh by about 70% with a 45 μM EGTA concentration, and the inhibition by EGTA was dose-dependent (Fig. 2A). Over the concentration of 90 μM EGTA, β-glucuronidase release was decreased almost to the basal (control) level. These results indicate that the increase in free calcium concentration is requisite for the CuPh-induced β-glucuronidase release. Figure 2B demonstrates that CuPh formed disulfide linkages and decreased the amount of free sulfhydyls in proteins. However, EGTA did not block the CuPh-mediated disulfide formation as shown

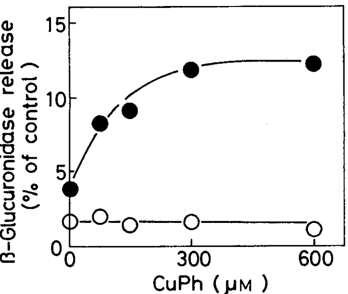

Fig. 1. Effect of CuPh on β-Glucuronidase Release in Permeable PMNs

Permeabilized PMNs (●) or intact PMNs (○) were incubated for 15 min at 37 °C with CuPh at the indicated concentrations. After the incubation, the PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as a percent release with respect to the total amount of β-glucuronidase in the PMN suspension. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and representative of three others.

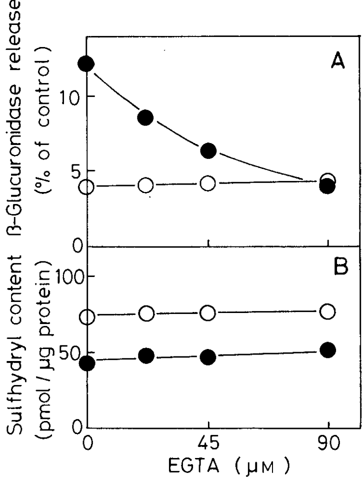

Fig. 2. Effects of EGTA on β-Glucuronidase Release (A) and Protein-Bound Free Sulfhydryls (B) in Permeable PMNs When Treated with CuPh

Permeabilized PMNs were incubated for 15 min with EGTA at the indicated concentrations in the absence (○) or presence (●) of 300 μM CuPh.

A: After the incubation, the PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as percent release with respect to the total amount of β-glucuronidase in the permeable PMN suspension.

B: The precipitated PMNs were used for the quantification of free sulfhydryls in protein. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of three others.

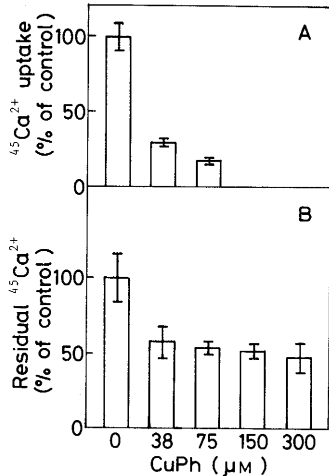

Fig. 4. Effects of CuPh on Active Uptake (A), and Passive Efflux (B) of Calcium

A: Permeabilized PMNs were incubated in the presence of 1.5 mM Mg/ATP for 30 min at 25 °C. The PMN suspensions contained CuPh at the concentrations indicated at abscissa. Calcium uptake was measured as described in the text, and expressed as the percent of control culture.

B: Permeabilized PMNs were incubated for 3.0 h at 25 °C in the presence of 1.7 mM Mg/ATP, then diluted 27 times with the reaction buffer containing 0.25 mM EGTA. After being incubated for 10 min, PMNs were collected on a membrane filter and washed with the same buffer to eliminate unincorporated Ca^{2+}. Experimental details were as described in Materials and Methods. Residual amounts of ^{45}Ca in permeable PMNs were determined. Data are means ± S.D. ($n=3$).

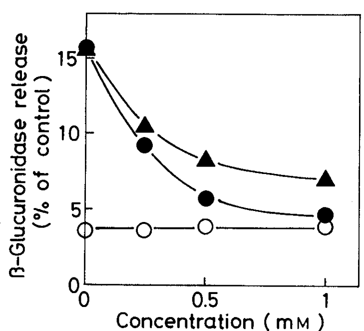

Fig. 3. Inhibitory Effect of DTT or W-7 on the CuPh-Induced β-Glucuronidase Release

Permeabilized PMNs were incubated in the absence (○) or presence (●, ▲) of 300 μM CuPh. The PMN suspensions contained DTT (○, ●) or W-7 (▲) at the concentrations indicated at abscissa. After incubation for 15 min at 37 °C, β-glucuronidase release was assessed. Results are presented from one experiment performed in duplicate, which is representative of two others.

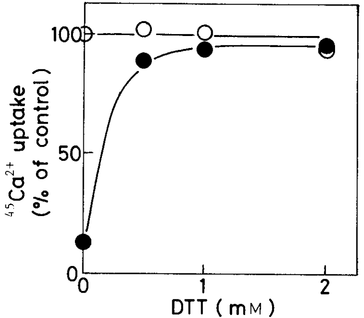

Fig. 5. Effect of DTT on the CuPh-Induced Inhibition of Calcium Uptake

DTT was added to permeabilized PMNs to give the indicated concentrations at abscissa with 75 μM CuPh (●) or without CuPh (○). After 30 min incubation at 25°C, active calcium uptake was assessed as in Fig. 4A. Results are presented from one experiment performed in duplicate, which is representative of two others.

in Fig. 2B. Protein-bound free sulfhydryl groups decreased by 34% with 300 μM CuPh concentration, and the extent of the decrease roughly correlated with that of the release of β-glucuronidase (data not shown). Thus, EGTA did not directly inhibit the oxidative reaction of CuPh.

Effects of DTT and W-7 on the β-Glucuronidase Release Induced by CuPh DTT, a sulfhydryl reducer, inhibited the β-glucuronidase release induced by CuPh dose-dependently as shown in Fig. 3. Half maximum inhibition of the CuPh-induced β-glucuronidase release was achieved by about 0.25 mM DTT. A DTT concentration of 1 mM completely inhibited the β-glucuronidase release from permeabilized PMNs, and the sulfhydryl quantity in proteins was recovered with DTT treatment (data not shown). W-7, an inhibitor for calmodulin, diminished the β-glucuronidase release from permeabilized PMNs elicited by CuPh. Half maximum inhibition was observed at the 300 μM concentration of W-7. Neither CuPh, W-7 nor DTT influenced the β-glucuronidase assay itself.

Effects of CuPh on Uptake and Efflux of Ca^{2+}, and Ca^{2+}-Dependent ATPase The inhibition of β-glucuronidase release by EGTA suggests that the release reaction by CuPh was mediated by an increase in cytoplasmic calcium concentration, so the effects of CuPh on calcium movements were examined (Fig. 4). CuPh inhibited Ca^{2+} uptake and promoted Ca^{2+} efflux from cytoplasmic Ca^{2+} store sites in permeable PMNs dose-dependently. CuPh resulted in 70% and 82% inhibition of Ca^{2+} uptake at 38 and 75 μM, respectively (Fig. 4A). The addition of 0.5 mM DTT, a disulfide reducing agent, recovered Ca^{2+} uptake almost to the control level even in the presence of 75 μM CuPh. The percent of Ca^{2+} uptake was not changed up to a concentration of 2 mM DTT alone (Fig. 5).

Ca^{2+} efflux was expressed as a percent of $^{45}Ca^{2+}$

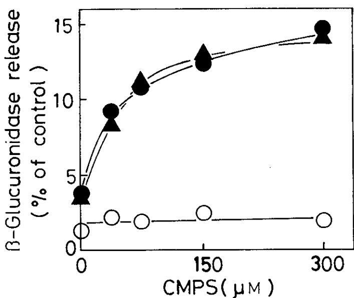

Fig. 6. Effect of CMPS on β-Glucuronidase Release in the Presence or Absence of EGTA in Permeable PMNs

Permeabilized PMNs were incubated for 15 min with CMPS (●, ▲) at the indicated concentrations in the absence (●) or presence (▲) of 0.25 mM EGTA. Intact PMNs were also treated with CMPS (○) as the control culture. After the incubation, PMNs were centrifuged, and the supernatant was obtained. Released β-glucuronidase in the supernatant was quantified and expressed as the percent release compared to the total amount of β-glucuronidase in the permeable PMN suspension. Experimental details were as described in Materials and Methods. Results are presented from one experiment performed in duplicate, and are representative of two others.

remaining in the intracellular Ca^{2+} store sites after incubation. CuPh promoted Ca^{2+} efflux from cytoplasmic Ca^{2+} store sites dose-dependently. The concentration of CuPh of 75 μM enhanced the $^{45}Ca^{2+}$ efflux and decreased the remaining $^{45}Ca^{2+}$ amount to about 50% of the control incubation (Fig. 4B).

CuPh-treated and washed permeable PMNs showed decreased Ca^{2+}-dependent ATPase activity. Ca^{2+}-ATPase activity was inhibited by approximately 82% and 90% with the addition of 75 and 150 μM CuPh, respectively. Specific activity of control cell suspension was 12.2 nmol/min/10^7 PMNs.

Effect of CMPS on Permeable PMNs CuPh catalyzes the air-oxidation of sulfhydryl groups to disulfides and is an efficient reagent to cross-link the sarcoplasmic reticulum Ca^{2+}-ATPase molecules. Thus, we examined whether disulfide cross-linking is essential for the β-glucuronidase release in permeable PMNs. Permeable PMNs were treated with CMPS, and the β-glucuronidase release was determined (Fig. 6). CMPS, which chemically modifies free sulfhydryl groups but does not form cross-links, was incorporated in the permeable PMNs which also released β-glucuronidase dose-dependently from permeabilized PMNs. However, this had no effect on intact PMNs. Both CMPS and CuPh released β-glucuronidase and decreased the amount of protein-bound free sulfhydryls (data not shown), but a difference in sensitivity to EGTA was found. EGTA did not inhibit the β-glucuronidase release induced by CMPS (Fig. 6). The mechanisms involved in the β-glucuronidase release elicited by these two sulfhydryl modifiers were thought to be different from each other.

Discussion

Artificially permeabilized PMNs are useful for both the examination of effects of ordinarily impermeable solutes and the investigation of signal transduction mechanisms. Saponin makes a complex (micelle) with a cholesterol on plasma membrane, and is able to selectively permeabilize the plasma membrane.[20] The saponin treatment of PMNs leaked approximately 50% of LDH and less than 10% of β-glucuronidase under our experimental conditions. β-Glucuronidase is a hydrolytic enzyme which is contained in a primary granule (azurophil),[21,22] and the release of β-glucuronidase represents the degranulation of PMNs. Smolen and Stoehr[23] reported that permeabilization of neutrophils was irreversible because the cells were still responsive to calcium ions and were trypan blue-positive even after washing out the saponin.[23] In permeabilized platelets, extracellularly added calcium elicited a release reaction.

PMNs play a major role in acute phase infections and immune responses and release various biologically-active substances in response to physiological stimulants. However, the function of intracellular sulfhydryl groups of proteins in the secretion reaction of PMNs is not yet well understood. Therefore, we wished to clarify whether or not sulfhydryl modifiers affect a lysozomal enzyme release in PMNs as in the case of platelets. The CuPh-induced β-glucuronidase release in permeable PMNs was inhibited by DTT. DTT reduces disulfide groups, so it is thought that DTT reduced disulfide bonds which were formed by CuPh-mediated cross-linking, and decreased the release of β-glucuronidase from permeable PMNs. We also demonstrated that CuPh, an air-oxidized-sulfhydryl agent, released β-glucuronidase from permeable PMNs but not from intact PMNs in the absence of exogeneous calcium ions, and the action was considerably inhibited by EGTA. However, the effect of CuPh on the cross-link formation of sulfhydryl groups was not inhibited by EGTA. Therefore, the action of CuPh was thought to be mediated by calcium ions indirectly. Target molecules of these sulfhydryl reagents must not be on the outer cell surface since intact PMNs did not respond at all to either CuPh or CMPS.

Since CuPh inhibits Ca^{2+}-ATPase of sarcoplasmic reticulumn[24] and H^+, K^+-ATPase of gastric vesicles[25] in cell-free systems, it is reasonable to assume that CuPh modified and inhibited Ca^{2+}-ATPase of permeabilized PMNs, blocked Ca^{2+} uptake, and accelerated Ca^{2+} leakage. An increase in cytoplasmic Ca^{2+} concentration is known to activate protein kinase C and calmodulin-dependent light chain kinase in platelets.[26-28] Actually, inhibition of Ca^{2+}-ATPase was observed in permeable PMNs treated with CuPh. CuPh also inhibited active calcium uptake, and enhanced calcium efflux from permeable PMNs. CuPh was suggested to work on the Ca^{2+}-dependent ATPase, causing leakage of Ca^{2+}, activating protein phosphorylation, and consequently releasing β-glucuronidase. Since W-7, a calmodulin inhibitor,[29] suppressed the CuPh-induced β-glucuronidase release from permeable PMNs, calmodulin might play a role in this release reaction. Based on these experimental results, the CuPh-induced β-glucuronidase release appeared to be closely related to intracellular calcium ion mobilization. In fact, β-glucuronidase was released by the addition of exogeneous calcium to the permeabilized PMNs (data not shown).

CMPS, a sulfhydryl modifying agent, reacts with free sulfhydryl groups without forming cross-links. CuPh but not CMPS influences the KCl conductance of gastric vesicles made from the parietal cells of hog gastric mucosa.[25] We wished to determine whether chemical cross-linking is a prerequisite for the elicitation of β-glucuronidase release in permeable PMNs. Since CMPS also released β-glucuronidase from permeable PMNs, cross-linking itself was not a prerequisite for the release reaction. Moreover, β-

glucuronidase release by CuPh and CMPS showed different sensitivities to inhibition by EGTA. EGTA blocked neither the decrease in free sulfhydryls induced by CuPh and CMPS nor the release of β-glucuronidase induced by CMPS. However, the β-glucuronidase release by CuPh was considerably inhibited by EGTA. These results suggest that the β-glucuronidase release by CMPS was not mediated by an elevation in calcium ion concentration. CMPS stimulated the incorporation of $^{32}PO_4$ from γ-^{32}P-ATP in total proteins and 70 kilodaltons band on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (unpublished data). It is possible that CMPS stimulated protein kinases directly and released β-glucuronidase even in the presence of EGTA.

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Aryloxy Acetic Acid Diuretics with Uricosuric Activity. I. Polycyclic Aryloxy Acetic Acids

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In order to obtain lead compounds for uricosuric diuretics, various polycyclic aryloxy acetic acids [isoindole derivative (7), quinazoline derivative (15), benzopyran derivative (20), xanthone derivative (24), benzofuran derivative (30) and indene derivative (36)] were prepared. These compounds were evaluated for diuretic activity in rats, uricosuric activity in rats and antihypertensive activity in 11-deoxycorticosterone acetate (DOCA)/salt hypertensive rats. Among the compounds, 20 showed potent diuretic, uricosuric and moderate antihypertensive activities. Therefore, we selected 20 as a lead compound for development of new uricosuric diuretics.

Keywords aryloxy acetic acid; 2,3-dihydro-1-oxo-1*H*-isoindole; 3,4-dihydro-4-oxo-3*H*-quinazoline; 4-oxo-4*H*-1-benzopyran; xanthone; benzo[*b*]furan; indene; ((5-chloro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy)acetic acid; diuretic; uricosuric; antihypertensive activity

The thiazides have a long history of effective control of a major segment of the hypertensive population. However, long-term administration of the thiazides results in side effects such as hyperuricemia, hypokalemia, and hyperglycemia. In the search for new diuretics which do not cause adverse reactions such as hyperuricemia, many compounds such as tienilic acid,[1] indacrinone,[2] S-8666,[3] and related compounds (IV,[4a] V,[4b] VI[4c]) have been examined, but few drugs have been marketed (Fig. 1).

We undertook research relating to acylphenoxyacetic acids in order to obtain a lead compound for the development of new uricosuric diuretics. In general, the compounds which are represented by formula VII show diuretic activities.[5] Molecular features found to result in potent diuretic and uricosuric activities for this series include the following: (1) Chloro substituents at positions 2 and 3 of the phenoxyacetic acid aromatic ring, a bulky substituent on the acyl group and formation of a bond between the α-position of the carbonyl group and position 5, as exemplified by indacrinone (MK-196),[2] or (2) a halogen substituent at position 2, a bulky substituent on the acyl group and introduction of a bioisoster instead of

I tienilic acid (SKF); II indacrinone (MK-196) (Merck); III S-8666 (Shionogi); IV (Merck); V HP-522 (Hoechst); VI 53385 (Abbott)

Fig. 1

VIII; VII; XII; IX; X; XI

Fig. 2

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Chart 1

Chart 2

Chart 3

the carbonyl group, with formation of a bond between the bioisoster and position 3, as exemplified by Hp-522.[4b)]

We therefore extended this annelation concept to the preparation of new ring-annelated compounds: the 2,3-dihydro-1-oxo-1*H*-isoindole derivative VIII (**7**: R = phenyl, X = Cl, Y = H), the 3,4-dihydro-4-oxo-3*H*-quinazoline derivative IX (**15**: R = phenyl, X = Cl, Y = H), the 4-oxo-4*H*-1-benzopyran derivative X (**20**: R = phenyl, X = Cl, Y = H), the xanthone derivative XI (**24**: X = Cl, Y = H), the benzofuran derivative XII (**30**: R = phenyl, Y = Br, Z = O) and the indene derivative XII (**36**: R = phenyl, Y = Cl, Z = CH_2).

These compounds were synthesized and tested for diuretic, uricosuric and antihypertensive activities. Herein we report the syntheses and biological activities of the newly designed compounds.

Chemistry The compounds prepared for this study are shown in Tables II—VI and their syntheses are outlined in Charts 1—6.

Synthesis of ((7-chloro-2,3-dihydro-1-oxo-2-phenyl-1*H*-isoindol-5-yl)oxy)acetic acid (**7**) was carried out by employing the reaction sequence outlined in Chart 1. Esterification of 2-chloro-6-methyl-4-nitrobenzoic acid (**1**), which was prepared from 2-methyl-4-nitroaniline *via* five reaction steps by the reported method,[6)] and successive reduction of the nitro group provided the aniline derivative (**3**) in high yield. Subsequently, the phenol (**4**) obtained by diazotization and hydrolysis from **3** was converted to the ester (**5**) by alkylation with ethyl bromoacetate/K_2CO_3. Bromination of **5** with *N*-bromosuccinimide (NBS) followed by ring closure with aniline gave ethyl ((7-chloro-2,3-dihydro-1-oxo-2-phenyl-1*H*-isoindol-5-yl)oxy)acetate (**6**) in low yield, and this was hydrolyzed to provide the desired compound (**7**).

((5-Chloro-3,4-dihydro-4-oxo-3-phenyl-3*H*-quinazolin-7-yl)oxy)acetic acid (**15**) was prepared as shown in Chart 2. The starting material (**8**) was chlorinated with sulfuryl chloride. The resulting aniline derivative (**9**) was diazotized by the method of Mallory[7)] and then treated with $Ni(CN)_2$, which was prepared by the reaction of $NiCl_2$ with NaCN, to give the benzonitrile derivative (**10**). Reaction of **10** with $AcOH/H_2SO_4$, diazotization, and successive degradation reaction led to the benzoic acid derivative (**11**). Chlorination of **11** with thionyl chloride, and reaction with aniline gave the anilide (**12**). Compound **12** was reduced with $SnCl_2$/concentrated HCl and then treated with formic acid

Chart 4

Chart 5

Chart 6

to yield 5-chloro-3,4-dihydro-7-methoxy-4-oxo-3-phenyl-3*H*-quinazoline (**13**). Ether cleavage of **13** with anhydrous $AlCl_3$, alkylation with ethyl bromoacetate/K_2CO_3, and subsequent hydrolysis gave the desired compound (**15**).

((5-Chloro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy)-acetic acid (**20**) was prepared by the route illustrated in Chart 3. Friedel–Crafts reaction at -10—-7 °C of 1-chloro-3,5-dimethoxybenzene (**16**) with phenylacetyl chloride and $AlCl_3$–$ZnCl_2$ and subsequent demethylation at 70 °C provided 1-(2-chloro-6-hydroxy-4-methoxyphenyl)-2-phenylethan-1-one (**17**) in moderate yield. Cyclization of **17** to form the benzopyran nucleus was carried out by the general method using ethyl orthoformate/pyridine/piperidine.[8] The resulting compound (**18**) was converted to **20** *via* 5-chloro-7-hydroxy-4-oxo-3-phenyl-4*H*-1-benzopyran (**19**) by a similar route to that described in Chart 2. On the other hand, Friedel–Crafts reaction at 5—10 °C of **16** with phenylacetyl chloride and $AlCl_3$–$ZnCl_2$ and subsequent demethylation at 65—70 °C gave 1-(4-chloro-2-hydroxy-6-methoxyphenyl)-2-phenylethan-1-one (**21**) as the main product, which was led to 7-chloro-5-hydroxy-4-oxo-3-phenyl-4*H*-1-benzopyran (**23**) (Chart 4) by a similar method to that described above. On the basis of the following findings, it is concluded that **19** has a 7-hydroxy group, and **23** has a 5-hydroxy group which can form an intramolecular hydrogen bond to the 4-carbonyl group. The infrared (IR) spectrum (KBr) of **23** showed a hydroxy absorption band at 3090 cm^{-1} attributable to the 5-OH group. On the other hand, the IR spectrum (KBr) of **19** showed a hydroxy absorption band at 3200 cm^{-1} attributable to the free phenolic OH group at position 7. In dilute solutions (0.0011 mmol/ml in $CDCl_3$), where intermolecular association is minimized, the signal of the 5-OH group on the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **23** was shifted to lower field (12.78 ppm) than that (6.04 ppm) of the 7-OH group of **19**. The comparison of **19** with **23** on thin-layer chromatography (TLC) showed that the *Rf* value of **23** was larger than that of **19** (see Experimental).

((1-Chloroxanthon-3-yl)oxy)acetic acid (**24**) was prepared according to the reported method.[9]

((7-Bromo-3-phenylbenzo[*b*]furan-6-yl)oxy)acetic acid (**30**) was prepared by means of the reaction sequence shown in Chart 5. Alkylation of 2-hydroxy-4-methoxy-benzophenone (**25**)[10] with ethyl bromoacetate/K_2CO_3, and subsequent treatment with 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) afforded the 6-methoxy-benzofuran (**26**). The ether cleavage of **26** with $AlCl_3$/EtSH[11] gave the 6-hydroxybenzofuran (**27**) in moderate yield. Bromination of **27** with equimolar bromine, and alkylation with ethyl bromoacetate/K_2CO_3, followed by hydrolysis produced the dicarboxylic acid (**29**) in high yield. Compound **29** was selectively decarboxylated by heating with $CuBr_2$ in Et_2NCHO to give the benzofuran derivative (**30**).

((7-Chloro-3-phenylinden-6-yl)oxy)acetic acid (**36**) was prepared according to the synthetic route depicted in

Chart 6. The benzaldehyde derivative (**31**), which was prepared as suggested by Ginsburg,[12] was treated with acetophenone under basic conditions to give the α,β-unsaturated ketone (**32**). Compound **32** which consisted of a mixture of (E) and (Z) isomers (the ratio was about 1 : 1) was hydrogenated by catalytic reduction to give the propanone derivative (**33**). A ring annelation to position 6 of **33** by using polyphosphoric acid (PPA) afforded the 6-methoxyindene (**34**), which was dealkylated by $AlBr_3$/$(Me)_2S$[13] to produce the hydroxy product (**35**). This compound was converted to the corresponding oxyacetic acid (**36**).

Biological Activities The compounds bearing an oxyacetic acid side chain prepared in this study were tested for diuretic and uricosuric activities in Wistar rats and for antihypertensive activity in 11-deoxycorticosterone acetate (DOCA)/salt hypertensive rats (see Experimental). The results are shown in Table I. Tienilic acid (TA), indacrinone (MK-196) and **24** were used as reference compounds.

(1) Diuretic Activity The excretion of urine after the administration of the reference compounds and the test compounds paralleled that of Na^+.

The diuretic activity of **20** was more potent than those of TA and **24**, and equipotent to that of MK-196. The diuretic activity of **7** was lower than that of **20**. On the other hand, **15**, **30** and **36** caused no detectable change in the excretion of urine.

(2) Uricosuric Activity Uric acid is the final product of purine metabolism in humans and some primates. However, in nonprimates such as rats and dogs, uric acid is further degraded to allantoin by uricase. It is therefore difficult to detect uricosuric activity in nonprimate animals. However, it was reported that uricosuric activity of TA was evaluated in rats by using the renal clearance method.[14] Similarly, in the present study, we observed that uricosuric agents such as TA and MK-196 produced increases in the fractional excretion rate of uric acid (FEua) without changing the concentration of uric acid in serum and urine.

The uricosuric activity (FEua) of **20** was more potent than those of TA, MK-196 and **24**. In contrast, **7** showed no uricosuric activity. Further investigations on the mechanisms of uricosuric action are in progress.

(3) Antihypertensive Activity In this study, the predrug value of systolic blood pressure (SBP) in DOCA/salt hypertensive rats was approximately 110 mmHg. In control rats, SBP subsequently increased to the level of 191 ± 6 mmHg ($n=8$), 3 weeks later.

The antihypertensive activity of **20** was equivalent to those of MK-196 and **24**, however, **7** was less active than MK-196.

From the biological evaluations described above, it was clear that **20** had the most favorable profile as a uricosuric diuretic. We therefore selected **20** as a lead compound for development of new uricosuric diuretics, and further investigations are in progress.

Table I. Biological Activities of Aryloxyacetic Acids

Compound No.	Diuretic[a)]	Uricosuric[b)]	Antihypertensive[c)]
7	+	−	+
15	−	NT	NT
20	+++	+++	+++
24	++	+	+++
30	−	NT	NT
36	−	NT	NT
TA	±	+	−
MK-196	+++	+	+++

a) Urine volume (ml/kg/5 h, 100 mg/kg, *p.o.* in rats): $-\leqq 0$, $0<\pm\leqq 10$, $10<+\leqq 20$, $20<++\leqq 30$, $30<+++$. *b)* Excretion of uric acid (%, FE_{ua}, 200 mg/kg, *p.o.* in rats): $-\leqq 0$, $0<+\leqq 50$, $50<++\leqq 150$, $150<+++$, NT; not tested. *c)* Systolic blood pressure (Δ mmHg, 100 mg/kg/d, *p.o.* in DOCA/salt hypertensive rats): $-\leqq 10$, $10<+\leqq 20$, $20<++\leqq 40$, $40<+++$, NT; not tested.

Experimental

Melting points are uncorrected. IR spectra were taken on a Hitachi 285 spectrometer. ^{1}H-NMR spectra were taken on Hitachi R40 and JNM-FX90Q spectrometers with tetramethylsilane (TMS) as an internal standard. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet), br (broad), m (multiplet). Chemical shifts are expressed in δ values and coupling constants are given in hertz. For column chromatography, Silica gel 60 (E. Merck, 0.063—0.200 mm) was used.

2-Chloro-6-methyl-4-nitrobenzoic Acid (1) The material (**1**) was prepared according to the reported method.[6b)]

Methyl 2-Chloro-6-methyl-4-nitrobenzoate (2) A solution of **1** (19.5 g, 90.4 mmol) in $SOCl_2$ (180 ml) was refluxed for 2 h and then excess $SOCl_2$ was evaporated off *in vacuo*. The resulting oily product was taken up in MeOH (200 ml), and the solution was refluxed for 4.5 h, then concentrated to precipitate crystals, which were collected by filtration to give **2** (17.8 g, 86%) (Table II).

Methyl 4-Amino-2-chloro-6-methylbenzoate (3) Compound **2** (31.4 g, 0.137 mol) in AcOEt (500 ml) was catalytically hydrogenated with PtO_2 (800 mg) under atmospheric pressure with absorption of 9.2 l of H_2 gas. After removal of the catalyst by filtration, the solvent was evaporated off *in vacuo*. The resulting residue was dissolved in a small amount of Et_2O and the insoluble product was filtered off. The filtrate was evaporated *in vacuo* to yield **3** (26.6 g, 97.4%) as pale yellow crystals (Table II).

Methyl 2-Chloro-4-hydroxy-6-methylbenzoate (4) Compound **3** (98 g, 0.49 mol) was dissolved in 10% H_2SO_4 (2.5 l) with heating at 70—80 °C and then a solution of (300 ml) of $NaNO_2$ (37.3 g, 0.53 mol) was added in small portions with stirring at the same temperature. After heating for an additional 1.5 h, the reaction mixture was cooled to room temperature and extracted with C_6H_6. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo* to give a residue. The resulting crude product was purified by column chromatography on silica gel with C_6H_6 as the eluent to provide **4** (77 g, 78%) as a brownish solid. ^{1}H-NMR ($CDCl_3$): 6.64 (1H, d, $J=2$), 6.50 (1H, d, $J=2$), 3.91 (3H, s), 2.23 (3H, s).

Ethyl ((3-Chloro-5-methyl-4-methoxycarbonylphenyl)oxy)acetate (5) A mixture of **4** (16.3 g, 81 mmol), ethyl bromoacetate (20.3 g, 0.122 mol) and K_2CO_3 (20.3 g, 0.122 mol) in *N,N*-dimethylformamide (DMF) (340 ml) was heated with stirring at 60—70 °C for 5 h. The solvent was evaporated off *in vacuo*, and the resulting oily residue was poured into ice-cooled H_2O, and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), and evaporated *in vacuo*. The resulting residue was purified by column chromatography on silica gel with C_6H_6 as the eluent to give **5** (19.9 g, 85.7%) as a brownish solid. ^{1}H-NMR ($CDCl_3$): 6.70 (1H, d, $J=2$), 6.60 (1H, d, $J=2$), 4.52 (2H, s), 4.22 (2H, q, $J=7$), 3.87 (3H, s), 2.30 (3H, s), 1.27 (3H, t, $J=7$). Compound **5** was used for the next reaction step without further purification.

Ethyl ((7-Chloro-2,3-dihydro-1-oxo-2-phenyl-1*H*-isoindol-5-yl)oxy)-acetate (6) A mixture of **5** (19.8 g, 69.1 mmol), NBS (14.8 g, 82.9 mmol) and benzoic peroxide (100 mg) in CCl_4 (500 ml) was refluxed for 6 h. After filtration to remove an insoluble product, a solution of aniline (64.3 g, 0.69 mol) in CCl_4 (200 ml) was added to the filtrate. The mixture was stirred for 1 h at room temperature, and then heated for an additional 1 h at 90—100 °C. After cooling, the reaction mixture was successively washed with 4 N HCl (520 ml) and H_2O, dried (Na_2SO_4), and then evaporated *in vacuo*. The resulting oily residue (18.8 g) was purified by column chromatography on silica gel with $CHCl_3$ as the eluent to give **6** (4.3 g, 18%) from **4** as white needles (Table II).

((7-Chloro-2,3-dihydro-1-oxo-2-phenyl-1*H*-isoindol-5-yl)oxy)acetic Acid (7) To a solution of **6** (4.2 g, 12.1 mmol) in DMF (40 ml), 10% NaOH (15 ml) was added in portions with stirring at room temperature. Stirring

Table II. ((7-Chloro-2,3-dihydro-1-oxo-2-phenyl-1*H*-isoindol-5-yl)oxy)acetic Acid (7) and Related Compounds

Compound No.	mp (°C) (Solv.)	Yield (%)	Analysis (%) Calcd (Found)			IR (KBr) (cm^{-1})	Solv.[b]	^{1}H-NMR Chemical shift (δ)
			C	H	N			
2	90—92 (MeOH)	86.0	$C_9H_8ClNO_4$			1730	C	8.00 (1H, d, J=3), 7.90 (1H, d, J=3), 3.95 (3H, s), 2.40 (3H, s)
			47.07	3.51	6.10			
			(46.63	3.47	6.11)			
3	74—77	97.4	$C_9H_{10}ClNO_2$			1700	C	6.44 (1H, d, J=3), 6.30 (1H, d, J=3), 3.87 (3H, s), 3.78 (2H, s), 2.23 (3H, s)
			54.14	5.05	7.02			
			(54.43	5.00	7.10)			
6	150—152 (EtOH)	18.0[a]	$C_{18}H_{16}ClNO_4$			1735, 1695	C	7.82—7.64 (2H, m), 7.46—7.01 (3H, m), 6.91—6.71 (2H, m), 4.65 (4H, s), 4.26 (2H, q, J=8), 1.31 (3H, t, J=8)
			62.52	4.66	4.05			
			(62.15	4.75	3.98)			
7	207—209 (DMF–MeCN)	83.0	$C_{16}H_{12}ClNO_3$			1700	D	7.82—7.72 (2H, m), 7.50—7.15 (3H, m), 7.15—7.00 (2H, m), 4.87 (2H, s), 4.82 (2H, s)
			60.10	3.78	4.38			
			(60.05	3.89	4.51)			

a) Yield from **5**. *b)* C=$CDCl_3$, D=DMSO-d_6.

was continued for 1 h, then the reaction mixture was poured into H_2O (300 ml), and acidified with concentrated HCl. The precipitated crystals were collected by filtration to give **7** (3.2 g, 82.7%) as white needles (Table II).

2-Chloro-4-methoxy-6-nitroaniline (9) Sulfuryl chloride (0.96 ml, 12 mmol) was added to a solution of **8** (2.0 g, 12 mmol) in acetic acid (200 ml) at room temperature. After being stirred for 30 min, the solution was poured into ice-cold H_2O, and the whole was evaporated *in vacuo* to yield a brownish solid, which was purified by column chromatography on silica gel with C_6H_6 as the eluent to give **9** (0.6 g, 25%) as pale yellow needles (Table III).

2-Chloro-4-methoxy-6-nitrobenzonitrile (10) A solution of $NiCl_2 \cdot 6H_2O$ (4.5 g, 18.9 mmol) in H_2O (15 ml) was added to a solution of NaCN (10.3 g, 0.21 mol) in H_2O (40 ml), followed by an Na_2CO_3 solution (40 g, 0.377 mol) in H_2O (75 ml), and then the reaction mixture was cooled at 5 °C with stirring. A solution of $NaNO_2$ (2.2 g, 31.9 mmol) in concentrated H_2SO_4 (16 ml) was added to a solution of **9** (5.43 g, 26.8 mmol) in acetic acid (50 ml) in portions with stirring at room temperature. The obtained diazonium salt was added to the previously prepared solution which contained $Ni(CN)_2$ over 1 h at the same temperature. The reaction mixture was stirred for 3 h, then a solution of Na_2CO_3 (40 g, 0.377 mol) in H_2O (100 ml) was added. The resulting product was collected by filtration, and purified by column chromatography on silica gel with C_6H_6 as the eluent to give **10** (3.54 g, 62%) (Table III).

2-Chloro-4-methoxy-6-nitrobenzoic Acid (11) A mixture of **10** (2.82 g, 13.3 mmol), acetic acid (100 ml), concentrated H_2SO_4 (100 ml) and H_2O (40 ml) was heated at 140 °C with vigorous stirring. The reaction temperature was lowered to 100 °C, then a solution of $NaNO_2$ (1.54 g, 22.3 mmol) in H_2O (15 ml) was added, and the mixture was stirred at the same temperature for an additional 30 min. After cooling, the reaction mixture was poured into ice-cold H_2O, and extracted with $CHCl_3$. The organic layer was washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo* to give **11** (2.39 g, 78%) (Table III).

2-Chloro-4-methoxy-6-nitrobenzanilide (12) A mixture of benzoic acid **11** (2.37 g, 10.2 mmol) and $SOCl_2$ (50 ml) was refluxed for 1 h. The excess $SOCl_2$ was evaporated off *in vacuo* to give the acid chloride, to which aniline (3.72 g, 40 mmol) in C_6H_6 (20 ml) was added, and the mixture was stirred at room temperature for 2 h. The precipitated crystals were collected by filtration to give the anilide **12** (2.99 g, 96%) (Table III).

5-Chloro-3,4-dihydro-7-methoxy-4-oxo-3-phenyl-3*H*-quinazoline (13) A solution of $SnCl_2 \cdot 2H_2O$ (67 g, 0.297 mol) in EtOH (90 ml) was added dropwise to a mixture of the anilide **12** (29 g, 94.6 mmol), EtOH (45 ml) and concentrated HCl (90 ml), and then the mixture was stirred at room temperature for 3 h. The precipitated crystals were collected by filtration, successively washed with H_2O, saturated Na_2CO_3 and H_2O, and dried to give 2-chloro-4-methoxy-6-aminobenzanilide. The filtrate was collected, made basic by adding 20% NaOH and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo* to give the aniline derivative as pale brown crystals. The combined yield of the aniline derivative was 22 g (84%). mp 159—160 °C (C_6H_6). IR (KBr) cm^{-1}: 1660 (-CONH-). ^{1}H-NMR (DMSO-d_6): 10.20 (1H, br s), 7.73—7.53 (2H, m), 7.40—6.90 (3H, m), 6.24 (2H, br s), 5.33 (2H, br s), 3.69 (3H, s).

A mixture of the aniline derivative (21 g, 75.9 mmol) and formic acid (500 ml) was refluxed for 2 h. Excess formic acid was evaporated off *in vacuo* to yield a residue which was purified by column chromatography on silica gel. The resulting residue was recrystallized from EtOH to give **13** (12.4 g, 57%) (Table III).

5-Chloro-3,4-dihydro-7-hydroxy-4-oxo-3-phenyl-3*H*-quinazoline (14) A mixture of **13** (12.4 g, 43.2 mmol) and anhydrous $AlCl_3$ (17.3 g, 0.13 mol) in C_6H_6 (400 ml) was refluxed with vigorous stirring for 2 h. After cooling, the organic layer was removed by decantation, and ice-cold H_2O was added to the resulting insoluble residue. The mixture was vigorously agitated. The resulting solid was collected by filtration, washed with hot H_2O, and then recrystallized from EtOH to give **14** (7.2 g, 61%) (Table III).

((5-Chloro-3,4-dihydro-4-oxo-3-phenyl-3*H*-quinazolin-7-yl)oxy)acetic Acid (15) A mixture of **14** (7.0 g, 25.7 mmol), ethyl bromoacetate (4.5 g, 26.9 mmol) and K_2CO_3 (4.2 g, 30.4 mmol) in DMF (150 ml) was heated at 60 °C with stirring for 2 h. Then 10% NaOH (20 ml) was added, and the mixture was stirred at the same temperature for an additional 1 h. After cooling, the reaction mixture was acidified with 10% HCl, and the precipitated crystals were collected by filtration to give **15** (6.5 g, 77%) (Table III).

1-(2-Chloro-6-hydroxy-4-methoxyphenyl)-2-phenylethan-1-one (17) A solution of **16** (60.4 g, 0.35 mol) in dry $ClCH_2CH_2Cl$ (140 ml) was added dropwise to a mixture of anhydrous $AlCl_3$ (56 g, 0.42 mol) and $ZnCl_2$ (5.72 g, 42 mmol) in dry $ClCH_2CH_2Cl$ (510 ml) with stirring at 0 °C. The mixture was cooled with stirring at −10 °C, then a solution of phenyl acetylchloride (59 g, 0.38 mol) in $ClCH_2CH_2Cl$ (120 ml) was added dropwise while maintaining the temperature at −10—−7 °C. After the addition, the reaction mixture was stirred at room temperature for 1 h, then heated at 70 °C for 1 h, poured into ice-cooled 20% HCl (1 l), and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4), and evaporated *in vacuo*. The resulting residue was recrystallized from C_6H_6 to give **17** (45.2 g, 46.7%) as white prisms (Table IV).

5-Chloro-7-methoxy-4-oxo-3-phenyl-4*H*-1-benzopyran (18) A mixture of **17** (1.11 g, 4 mmol), ethyl orthoformate (7.6 ml), dry pyridine (10 ml) and dry piperidine (0.4 ml) was refluxed for 4 h. The reaction mixture was poured into ice-cooled 20% HCl (100 ml), and the insoluble material was extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried (Na_2SO_4), and then concentrated to dryness *in vacuo*. The resulting residue was purified by silica gel (50 g) column chromatography with C_6H_6 as the eluent to give **18** (0.53 g, 64%) (Table IV).

5-Chloro-7-hydroxy-4-oxo-3-phenyl-4*H*-1-benzopyran (**19**) was prepared from **18** in a similar manner to that used for the preparation of **14** from **13** (Table IV). ^{1}H-NMR (0.0011 mmol/ml in $CDCl_3$): 7.83 (1H, s, 2-H), 7.53—7.37 (5H, m, arom. H), 6.95 (1H, d, J=2.5, 6-H or 8-H), 6.80 (1H, d, J=2.5, 6-H or 8-H), 6.04 (1H, s, 7-OH).

((5-Chloro-4-oxo-3-phenyl-4*H*-1-benzopyran-7-yl)oxy)acetic acid (**20**) was prepared from **19** in a similar manner to that used for the preparation of **15** from **14** (Table IV).

1-(4-Chloro-2-hydroxy-6-methoxyphenyl)-2-phenylethan-1-one (21) A solution of phenyl acetylchloride (16.8 g, 0.109 mol) in dry $ClCH_2CH_2Cl$

TABLE III. ((5-Chloro-3,4-dihydro-4-oxo-3-phenyl-3H-quinazolin-7-yl)oxy)acetic Acid (**15**) and Related Compounds

Compound No.	mp (°C) (Solv.)	Yield (%)	Analysis (%) Calcd (Found) C	H	N	IR (KBr) (cm^{-1})	Solv.[a]	^{1}H-NMR Chemical shift (δ)
9	115—116 (C_6H_6)	25.0	$C_7H_7ClN_2O_3$			3480, 3370 1500, 1210	D	7.45 (2H, s), 6.90 (2H, br s), 3.75 (3H, s)
			41.50	3.48	13.83			
			(41.60	3.44	14.03)			
10	127—128	62.0	$C_8H_5ClN_2O_3$			2220	C	7.62 (1H, d, J=3), 7.26 (1H, d, J=3), 3.95 (3H, s)
			45.20	2.37	13.18			
			(45.33	2.50	13.19)			
11	158—160	78.0	$C_8H_6ClNO_5$			1710	C–D	7.52 (1H, d, J=3), 7.26 (1H, d, J=3), 3.92 (3H, s)
			41.49	2.61	6.05			
			(41.59	2.50	6.07)			
12	188—190	96.0	$C_{14}H_{11}ClN_2O_4$			1650	D	10.55 (1H, br s), 7.80—7.50 (4H, m), 7.45—6.95 (3H, m), 3.93 (3H, s)
			54.83	3.61	9.13			
			(55.20	3.75	9.15)			
13	175—176 (EtOH)	48.0	$C_{15}H_{11}ClN_2O_2$			1690	D	8.22 (1H, s), 7.47 (5H, s), 7.12 (1H, d, J=3), 7.05 (1H, d, J=3), 3.90 (3H, s)
			62.84	3.87	9.77			
			(63.13	3.98	9.81)			
14	>280 (EtOH)	61.0	$C_{14}H_9ClN_2O_2$			1690	D	10.88 (1H, br s), 8.15 (1H, s), 7.46 (5H, s), 6.98 (1H, d, J=2), 6.90 (1H, d, J=2)
			61.66	3.33	10.27			
			(61.57	3.42	10.13)			
15	258—259 (EtOH)	77.0	$C_{16}H_{11}ClN_2O_4$			1700	D	8.23 (1H, s), 7.47 (5H, m), 7.17 (1H, d, J=3), 7.04 (1H, d, J=3), 4.88 (2H, s)
			58.11	3.35	8.47			
			(58.41	3.44	8.46)			

a) D=DMSO-d_6, C=$CDCl_3$.

TABLE IV. ((5-Chloro-4-oxo-3-phenyl-4H-1-benzopyran-7-yl)oxy)acetic Acid (**20**) and Related Compounds

Compound No.	mp (°C) (Solv.)	Yield (%)	Analysis (%) Calcd (Found) C	H	IR (KBr) (cm^{-1})	Solv.[b]	^{1}H-NMR Chemical shift (δ)
17	123—125 (C_6H_6)	46.7	$C_{15}H_{13}ClO_3$		1610, 1320, 1210, 1150	C	12.89 (1H, s), 7.40—7.00 (5H, m), 6.50 (1H, d, J=3), 6.30 (1H, d, J=3), 4.50 (2H, s), 3.78 (3H, s)
			65.10	4.74			
			(65.26	4.80)			
18	173—174 (C_6H_6)	64.0	$C_{16}H_{11}ClO_3$		1635	C	7.81 (1H, s), 7.60—7.20 (5H, m), 6.96 (1H, d, J=2), 6.75 (1H, d, J=2), 3.86 (3H, s)
			67.02	3.87			
			(67.39	4.02)			
19	284—286 (MeOH)	96.3	$C_{15}H_9ClO_3$		3200, 1630, 1590, 1270	D	11.05 (1H, s), 8.20 (1H, s), 7.60—7.20 (5H, m), 6.90 (1H, d, J=2), 6.80 (1H, d, J=2)
			67.02	3.87			
			(67.33	4.02)			
20	242—245 (MeCN)	73.0[a]	$C_{17}H_{11}ClO_5$		1735, 1640	D	8.37 (1H, s), 7.60—7.30 (5H, m), 7.16 (2H, s), 4.93 (2H, s)
			61.74	3.35			
			(61.64	3.43)			
21	74—75 (C_6H_6)	54.4	$C_{15}H_{13}ClO_3$		3020, 1630, 1590, 1210	C	7.70—7.40 (5H, m), 6.55 (1H, d, J=2), 6.32 (1H, d, J=2), 4.30 (2H, s), 3.85 (3H, s)
			65.10	4.74			
			(65.24	4.80)			
22	91—93 (EtOH)	73.9	$C_{16}H_{11}ClO_3$		1660	C	7.71 (1H, s), 7.48—7.12 (5H, m), 6.93 (1H, d, J=3), 6.69 (1H, d, J=3), 3.89 (3H, s)
			67.02	3.87			
			(67.01	3.96)			
23	142—143 (EtOH)	91.7	$C_{15}H_9ClO_3$		3090, 1650, 1060	D	12.91 (1H, s), 8.62 (1H, s), 7.60—7.42 (5H, m), 6.96 (2H, s)
			66.06	3.34			
			(65.80	3.33)			

a) Yield from the corresponding hydroxy compound. *b*) C=$CDCl_3$, D=DMSO-d_6

(40 ml) was added dropwise to a mixture of **16** (17.2 g, 0.1 mol), anhydrous $AlCl_3$ (16 g, 0.12 mol) and $ZnCl_2$ (1.64 g, 12 mmol) in dry $ClCH_2CH_2Cl$ (140 ml) with stirring at 5—10 °C. The reaction mixture was stirred at room temperature for an additional 1 h, and then heated at 65—70 °C for 3 h, poured into ice-cooled 4 N HCl (800 ml) and extracted with Et_2O. The extract was washed with H_2O, dried (Na_2SO_4), and then evaporated to give an oily residue (28.3 g). Petroleum ether (300 ml) was added to a solution of the resulting residue in C_6H_6 (30 ml) to precipitate white prisms. The crystals were collected by filtration to yield **21** (7.41 g). The filtrate was evaporated, and the resulting residue was purified by silica gel (300 g) column chromatography with C_6H_6–petroleum ether (1 : 1, v/v) as the eluent to give **21** (7.64 g). The total yield of **21** was 15.05 g (54.4%) (Table IV).

7-Chloro-5-methoxy-4-oxo-3-phenyl-4H-1-benzopyran (**22**) was prepared from **21** in a similar manner to that used for the preparation of **18** from **17** (Table IV).

7-Chloro-5-hydroxy-4-oxo-3-phenyl-4*H*-1-benzopyrane (23) A mixture of **22** (1.43 g, 5 mmol) and anhydrous $AlCl_3$ (0.80 g, 6 mmol) in C_6H_6 (20 ml) was heated at 60 °C with stirring for 1 h. After cooling, the reaction mixture was poured into ice-cold H_2O and extracted with $CHCl_3$. The extract was washed with H_2O, dried (Na_2SO_4), and then evaporated *in vacuo*. The resulting yellow residue was purified by column chromatography on silica gel with C_6H_6 as the eluent. The obtained product was recrystallized from EtOH to give **23** (1.25 g, 91.7%) (Table IV). ^{1}H-NMR

Table V. ((7-Bromo-3-phenyl-benzo[*b*]furan-6-yl)oxy)acetic Acid (**30**) and Related Compounds

Compound No.	mp (°C) (Solv.)	Yield (%)	Analysis (%) Calcd (Found)		IR (KBr) (cm^{-1})	Solv.[a)]	^1H-NMR Chemical shift (δ)
			C	H			
27	161—163 (C_6H_6)	65.0	$C_{17}H_{14}O_4$ 72.33 (72.66	5.00 5.11)	1690	D	10.16 (1H, s), 7.80—7.30 (6H, m), 7.06 (1H, d, J=2), 6.88 (1H, dd, J=2, 9), 4.23 (2H, q, J=8), 1.20 (3H, t, J=8)
28	153—155 (C_6H_6)	74.0	$C_{17}H_{13}BrO_4$ 56.53 (56.41	3.63 3.65)	1710	D	10.93 (1H, s), 7.80—7.30 (5H, m), 7.37 (1H, d, J=9), 7.06 (1H, d, J=9), 4.25 (2H, q, J=8), 1.20 (3H, t, J=8)
30	161—162 (MeCN)	66.0	$C_{16}H_{11}BrO_4$ 55.36 (55.18	3.19 3.21)	1700	D	8.32 (1H, s), 7.78 (1H, d, J=9), 7.70—7.20 (5H, m), 7.05 (1H, d, J=9), 4.86 (2H, s)

a) DMSO-d_6.

(0.0011 mmol/ml in $CDCl_3$): 12.78 (1H, s, 5-OH), 7.97 (1H, s, 2-H), 7.53—7.40 (5H, m, arom. H), 6.97 (1H, d, J=1.5, 6-H or 8-H), 6.85 (1H, d, J=1.5, 6-H or 8-H).

Rf value for **19** on TLC (E. Merck, Art 5714, Kieselgel 60 F_{254}) using $CHCl_3$ as the developing solvent was 0.05. On the other hand, that of **23** was 0.50.

Ethyl 6-Methoxy-3-phenylcoumarilate (26) A mixture of **25** (76 g, 0.33 mol), ethyl bromoacetate (58.5 g, 0.35 mol) and K_2CO_3 (92 g, 0.67 mol) in Me_2CO (1 l) was refluxed for 4 d. The precipitated insoluble product was filtered off, and then the filtrate was evaporated *in vacuo* to give a residue. The obtained residue was dissolved in C_6H_6, washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo* to give the ester (96.0 g, 92%) as an oily product. A solution of the ester (95 g, 0.304 mol) and DBU (22 ml) in C_6H_6 (200 ml) was dehydrated by using a Dean and Stark water separator under reflux for 4 d. The reaction mixture was washed with H_2O, and then the C_6H_6 layer was evaporated *in vacuo*. The resulting product (79.85 g) was recrystallized from C_6H_6–petroleum ether to give **26** (57.5 g, 64%) as pale yellow prisms. mp 87—89 °C. IR (KBr) cm^{-1}: 1710 (–COOEt). ^1H-NMR ($CDCl_3$): 7.80—7.25 (6H, m), 7.03 (1H, d, J=2), 6.86 (1H, dd, J=2, 9), 4.25 (2H, q, J=8), 3.75 (3H, s), 1.24 (3H, t, J=8).

Ethyl 6-Hydroxy-3-phenylcoumarilate (27) Ethanethiol (75.6 g, 1.22 mol) was added dropwise to a suspension of anhydrous $AlCl_3$ (54.26 g, 0.41 mol) in $ClCH_2CH_2Cl$ (300 ml) under cooling with ice-cold H_2O. After the anhydrous $AlCl_3$ had dissolved, a solution of **26** (40 g, 0.136 mol) in $ClCH_2CH_2Cl$ (150 ml) was added dropwise at the same temperature. After being stirred for an additional 5 h, the reaction mixture was poured into ice-cold H_2O, and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo*. The resulting solid was recrystallized from C_6H_6 to give **27** (25.0 g, 65%) (Table V).

Ethyl 7-Bromo-6-hydroxy-3-phenylcoumarilate (28) A solution of bromine (5.70 g, 35.6 mmol) in acetic acid (200 ml) was added dropwise to a solution of **27** (10 g, 35.6 mmol) in acetic acid (500 ml) with stirring at room temperature. The reaction mixture was poured into H_2O, and then the precipitated product was collected by filtration. The obtained crude **28** (11.7 g) was recrystallized from C_6H_6 to give **28** (9.70 g, 74%) (Table V).

((7-Bromo-2-carboxy-3-phenyl-benzo[*b*]furan-6-yl)oxy)acetic Acid (29) Compound **29** was prepared from **28** in a similar manner to that described for the synthesis of **15**. mp 218—219 °C. IR (KBr) cm^{-1}: 1690 (–COOH). ^1H-NMR (DMSO-d_6): 7.70—7.30 (6H, m), 7.08 (1H, d, J=9), 4.90 (2H, s).

((7-Bromo-3-phenylbenzo[*b*]furan-6-yl)oxy)acetic Acid (30) A mixture of **29** (21 g, 41 mmol) and copper(II) bromide (6.0 g, 27 mmol) in $(Et)_2NCHO$ (300 ml) was refluxed for 3 h. After removal of the solvent, the resulting residue was dissolved in AcOEt (100 ml) and the insoluble product was filtered off. The filtrate was acidified with 3 N HCl and evaporated *in vacuo*. The resulting residue was dissolved in Et_2O (300 ml), and the insoluble product was filtered off. To the filtrate was added 10% NaOH (200 ml), and the mixture was shaken vigorously. The separated aqueous layer was adjusted to pH 2 with 4 N HCl and the precipitated crystals were collected by filtration to give **30** (40 g, 66%) as pale yellow prisms (Table V).

3-(2-Chloro-3-methoxyphenyl)-1-phenyl-2-propen-1-one (32) A 10% NaOH solution (50 ml) was added in portions to a solution of **31** (23.7 g, 0.139 mol) and acetophenone (16.7 g, 0.139 mol) in EtOH (226 ml) with stirring. Vigorous stirring was continued at room temperature for 30 min, then the precipitated crystals were collected by filtration and washed with H_2O to yield a crude product (35.8 g), which was recrystallized from EtOH to give **32** (24.2 g, 63.4%) as white prisms. ^1H-NMR ($CDCl_3$) δ: (*E*) isomer: 8.24 (1H, d, J=16), 7.17 (1H, d, J=16). (*Z*) isomer: 6.83 (1H, d, J=9). The product, which consisted of (*E*) and (*Z*) isomers (ratio = 1 : 1), was used for the next step without isolation of the (*E*) and (*Z*) isomers.

3-(2-Chloro-3-methoxyphenyl)-1-phenylpropan-1-one (33) The mixture (13.6 g, 50 mmol) of (*E*) and (*Z*) isomers **32** in AcOEt (150 ml) was catalytically hydrogenated with 10% Pd/C (700 mg) under atmospheric pressure with absorption of 1.12 l of H_2 gas. After removal of the catalyst by filtration, the solvent was evaporated off *in vacuo*. The resulting oily residue (14.5 g) was purified by column chromatography on silica gel to give 33 (8.82 g, 64%) (Table VI).

7-Chloro-6-methoxy-3-phenylindene (34) A mixture of **33** (15 g, 55.6 mmol) and PPA (250 g) was agitated vigorously at 80—90 °C for 50 min. The reaction mixture was poured into ice-cold H_2O, stirred vigorously and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The resulting residue was recrystallized from MeOH to give **34** (10.4 g, 74.5%) (Table VI).

7-Chloro-6-hydroxy-3-phenylindene (35) Compound **34** (0.26 g, 1 mmol) was added to a solution of $AlBr_3$ (0.67 g, 2.5 mmol) in CH_2Cl_2 (5 ml) and $(Me)_2S$ (5 ml), and then stirred at room temperature for 24 h. The reaction mixture was poured into ice-cold H_2O and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with H_2O, and then evaporated to dryness *in vacuo*. The resulting oily residue (0.267 g) was purified by column chromatography on silica gel. The obtained crude product was recrystallized from C_6H_6 to give **35** (0.169 g, 88.5%) (Table VI).

((7-Chloro-3-phenylindene-6-yl)oxy)acetic Acid (36) A mixture of **35** (11.0 g, 45.7 mmol), ethyl bromoacetate (22.9 g, 0.137 mol), powdered KOH (7.68 g, 0.137 mol) in dried EtOH (680 ml) was stirred at room temperature for 24 h. The reaction mixture was poured into ice-cooled 4 N HCl (350 ml) and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), and then evaporated to dryness *in vacuo*. The resulting residue was recrystallized from EtOH to give the ester (8.4 g, 62.9%). mp 100—102 °C.

A solution of KOH (7.01 g, 0.125 mol) in H_2O (250 ml) was added to a solution of the ester (8.2 g, 0.025 mol) in MeOH (800 ml) and the mixture was kept at room temperature for 1 h. After concentration of the reaction mixture to one-fifth, the basic solution was added to ice-cold H_2O (100 ml), then the mixture was acidified with 4 N HCl and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), and evaporated *in vacuo*. The resulting residue was recrystallized from MeCN to give **36** (7.0 g, 93.8%) (Table VI).

Diuretic and Natriuretic Activities Male Wistar rats weighing 120—180 g were starved for 18 h and deprived of drinking water for 2 h before the test. The animals were orally loaded with 25 ml/kg of physiological saline, immediately followed by oral administration of the test drugs which were suspended in a 0.5% aqueous solution of carboxymethylcellulose sodium (CMC). The rats were housed singly in stainless steel metabolic cages with no access to food or water. Urine was collected during the 5-h period after dosing, and urine volume was measured. Urinary sodium and potassium contents were estimated by using an

TABLE VI. ((7-Chloro-3-phenylinden-6-yl)oxy)acetic Acid (**36**) and Related Compounds

Compound No.	mp (°C) (Solv.)	Yield (%)	Analysis (%) Calcd (Found)		IR (KBr) (cm^{-1})	Solv.[a]	^{1}H-NMR Chemical shift (δ)
			C	H			
33	58—60 (EtOH)	64.0	$C_{16}H_{15}ClO_2$ 69.94 (70.26	5.50 5.64)	1690	C	8.10—7.80 (2H, m), 7.60—6.65 (6H, m), 3.85 (3H, s), 3.20 (4H, s)
34	133—134 (MeOH)	74.5	$C_{16}H_{13}ClO$ 74.85 (74.60	5.10 5.05)	3000, 1480, 1280, 1060	C	7.70—7.20 (6H, m), 6.93 (1H, d, J=9), 6.51 (1H, t, J=2), 3.97 (3H, s), 3.55 (2H, d, J=2)
35	138—139 (C_6H_6)	88.5	$C_{15}H_{11}ClO$ 74.23 (74.50	4.57 4.58)	3370	C	7.60—7.20 (6H, m), 6.89 (1H, d, J=8), 6.33 (1H, t, J=3), 5.36 (1H, s), 3.36 (2H, d, J=3)
36	159—160 (MeCN)	58.6	$C_{17}H_{13}ClO_3$ 67.89 (68.16	4.36 4.55)	1720	C	7.65—7.30 (5H, m), 7.28 (1H, d, J=9), 6.86 (1H, br s), 6.84 (1H, d, J=9), 6.50 (1H, t, J=2), 4.74 (2H, s), 3.50 (2H, d, J=2)

a) C=$CDCl_3$.

electrolyte analyzer with ion-selective electrodes (PVA-4, Photovolt, U.S.A.).

Uricosuric Activity Male Wistar rats weighing 180—220 g were starved for 18 h and deprived of drinking water for 2 h before the test. The animals were orally given both 25 ml/kg of saline and the test drugs. Sixty minutes after dosing of the test drugs, the animals were housed singly in the metabolic cages, and urine was collected for 30 min. Immediately after the 30-min collection of urine, blood was taken from the carotid artery under ether anesthesia. At the same time, the remaining urine in the bladder was directly collected by using a syringe, and total urine volume was measured. A blood sample was centrifuged within 30 min after collection, and resultant plasma was used for the measurement of uric acid and creatinine. Plasma and urinary uric acid were estimated by the uricase method (Uric acid β-test, Wako, Osaka, Japan), and creatinine content was determined by Jaffe's method (Creatinin Set, Wako, Osaka, Japan). FEua was calculated by using the following formula: FEua = C_{ua}/C_{cr}, where C_{ua} is uric acid clearance and C_{cr} is creatinine clearance.

Antihypertensive Activity Four-week-old male rats (Sprague–Dawley) weighing 150—180 g were used. The left kidney of each rat was removed aseptically under ether anesthesia. From one week after the unilateral nephrectomy, the animals were treated with DOCA (15 mg/kg, s.c., once a week) and received 1% sodium chloride as drinking water.[15] Simultaneously, the test drugs were orally administered to the animals daily for 3 weeks. SBP of the animals in a conscious state was measured weekly prior to the daily dosing, by a tail cuff method.[16]

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Effect of Autoxidation of Hydrogenated Castor Oil Containing 60 Oxyethylene Groups on Degradation of Miconazole

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When 30 mg/ml aqueous hydrogenated castor oil consisting of 60 oxyethylene group (HCO-60) solutions were shaken under fluorescent light of about 500 lux in a water bath at 30 °C or 60 °C, hydroperoxide, formaldehyde, and formic acid were formed. An aqueous solution containing 10 mg/ml miconazole (MCZ), 100 mg/ml HCO-60, and 1 mg/ml lactic acid was sealed in ampoules and stored at various temperatures for 8 d. At 40 °C or above, the concentration of MCZ decreased accompanied by a decrease in pH and formaldehyde formation. However, in the presence of nitrogen, no degradation was observed. The degradation of MCZ was also observed in the presence of iron(II) ion and hydrogen peroxide. Degradation of MCZ and HCO-60 was prevented by the addition of hydroxy radical scavengers, especially potassium iodide and thiourea.

These results indicate that hydroxy radicals generated by autoxidation of HCO-60 degraded MCZ.

Keywords hydrogenated castor oil; ethylene oxide chain; miconazole; degradation; hydroxy radical; autoxidation; hydroperoxide; formaldehyde; surfactant

Polyoxyethylene surfactants, especially polysorbates, polyoxyethylene castor oil and hydrogenated castor oil (HCO), are frequently used as solubilizing agents for injections. These compounds have been considered as stable except for the hydrolysis of esters. However, autoxidation, and degradation of the ethylene oxide (EO) chain have been reported under certain conditions of light, temperature, or catalysis by metals.[1] We therefore studied the effect of light and temperature on the autoxidation of HCO consisting of 60 oxyethylene groups (HCO-60). We also studied the effect of HCO-60 degradation products on miconazole (MCZ) stability. MCZ, an imidazole antifungal agent, was selected as it is relatively stable when injected with HCO-60.

Materials and Methods

Materials HCO-60, polysorbate 20 (PLS-20), polyethylene glycol 400 (PEG-400), and laureth 9 (hydroxypolyethoxydodecane, LAR-9) was kindly provided by Nikko Chemical Co., Ltd. (Tokyo). The figures indicate the approximate EO number in a molecule. MCZ was kindly provided by Mochida Pharmaceutical Co., Ltd. (Tokyo). 6-Hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox) was obtained from Aldrich Chemical Co., Inc., U.S.A. All other chemicals were of the highest purity available, and used without further purification.

Autoxidation Conditions of HCO-60 and PLS-20 Aqueous Solutions Under fluorescent light of about 500 lux, 50 ml of 30 mg/ml aqueous HCO-60 and PLS-20 solutions were shaken in a water bath at 30 °C or 60 °C, and stored without loss of solvent by evaporation.

Conditions of Degraded HCO-60 (D-HCO-60) Under the above conditions, HCO-60 solution was shaken at 60 °C for 3 d. After incubation, the concentrations of hydroperoxide (HPO) and formaldehyde (FA) were 1.1 and 3.8 mM, respectively.

Storage Conditions of MCZ Unless otherwise specified, samples for storage were aqueous solutions containing 10 mg/ml MCZ, 100 mg/ml HCO-60, and 1 mg/ml lactic acid, or half of these concentrations in a final volume of 1 ml, adjusted to pH 4.4 with sodium hydroxide or hydrochloric acid sealed in ampoules. These samples were incubated in a water bath for 8—16 d at 30 to 60 °C in the dark. The inner volume of the ampoules used was 3.5 and 9.0 ml, and the volume ratio of the sample solution to air in the ampoules was 0.51 and 0.13, respectively.

Determination of HPO, H_2O_2, FA, and Formic Acid HPO and H_2O_2 in micellar solutions were determined by the iodimetric spectrophotometric method of Hamburger *et al.*[1a] Appropriate volumes of the samples were diluted to contain 1% HCO-60 or 1% PLS-20, 5% KI, and 0.1 M phosphate buffer (pH 6.0). The time of the addition of KI to the sample was taken as time zero, and the absorbance at 365 nm of the iodine released was determined for 30 min at 5-min intervals. The values were plotted on a logarithmic scale, and the absorbance of time zero was estimated by extrapolation. FA was determined by adding 4.5 ml of acetylacetone solution, which contained 150 g of ammonium acetate, 3 ml of acetic acid and 2 ml of acetylacetone per liter, to 0.5 ml of the sample and measuring the absorbance at 412 nm after incubation for 60 min at 60 °C. Formic acid was determined by the spectrophotometric method of Lang *et al.*[2] One milliliter of a solution, which contained 0.5 g of citric acid monohydrate and 10 g of acetamide in 100 ml isopropanol, 0.04 ml of 30% sodium formate, and 3.5 ml of acetic anhydride, was added to 0.5 ml of the sample, and the absorbance at 515 nm was determined.

Determination of the Residual Amount of MCZ MCZ was determined by high performance liquid chromatography (HPLC). To 70 μl of the sample were added 0.85 mg of clotrimazole as an internal standard and 2.0 ml of a mobile phase described below. The mixture (20 μl) was injected into a HPLC column. The residual amount of MCZ was calculated from the MCZ amount of the initial condition. The HPLC conditions were as follows: apparatus; Waters model 600 multi-solvent delivery system, model 490 variable wavelength UV detector and model 741 data analyzer, column; μ Bondasphere 5 μ C_{18}—100 Å 3.9 × 150 mm maintained at 40 °C, mobile phase; 0.05 M ammonium phosphate–methanol (15 : 85, v/v), flow rate; 0.7 ml/min, wave length of detector; 272 nm.

Ratio of the Degradation Product to MCZ The ratio (R) of degradation product to MCZ was calculated according to the equation:

$$R=\frac{C}{A}$$

Where C is the peak area of the main degradation product of MCZ, and A is the peak area of MCZ, at 254 nm. C and A correspond to the peaks C and 2 in Fig. 2, respectively.

Statistical Analysis The experimental data was tested for statistically significant differences by means of the Student's t test.

Results

Autoxidation of HCO-60 and PLS-20 Aqueous Solutions Figure 1 shows changes in the amount of HPO produced at 30 °C or 60 °C. At 60 °C, the reaction reached the propagation step early and progressed to the termination step with both HCO-60 and PLS-20. At 30 °C, the reaction remained at the initiation step even after 50 d, but the HPO concentration increased gradually. Table I shows the pH, FA concentration and formic acid concentration. At 30 °C, changes were slightly observed, and marked changes occurred at 60 °C.

The Effect of Temperature and Gaseous Phase on the Stability of MCZ Solutions Containing HCO-60 Figure 2 shows the chromatograms of MCZ mixtures containing HCO-60 before and after incubation for 8 d at 60 °C. After

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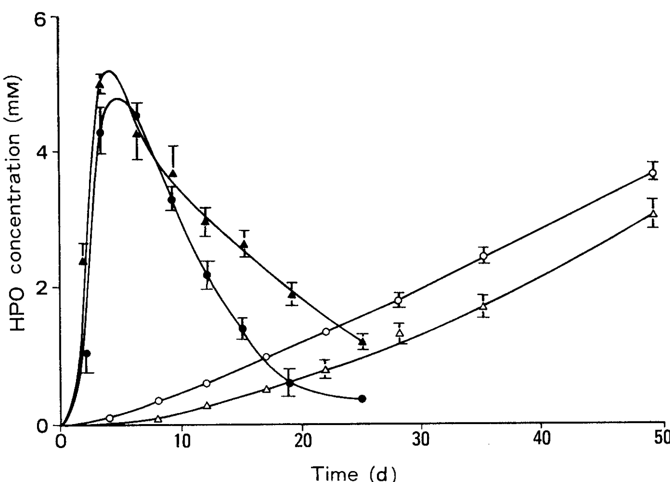

Fig. 1. HPO Formation in HCO-60 and PLS-20 Aqueous Solutions

Under fluorescent light of about 500 lux, 50 ml of 30 mg/ml aqueous HCO-60 (△, ▲) and PLS-20 (○, ●) solutions were shaken in a water bath at 30 or 60 °C. Open symbols, at 30 °C; closed symbols, at 60 °C. Each value represents the mean ± S.D. of 3 experiments.

Table I. The Effect of Autoxidation on HCO-60 and PLS-20 Aqueous Solutions

Surfactant	Storage temperature (°C)	pH	FA concentration (mM)	Formic acid concentration (mM)
HCO-60	Before storage (control)	6.06 ± 0.06	0.02 ± 0.01	0.04 ± 0.01
	30	4.33 ± 0.12[a]	0.13 ± 0.01[a]	1.01 ± 0.06[a]
	60	2.38 ± 0.01[a]	7.45 ± 0.40[a]	45.70 ± 0.86[a]
PLS-20	Before storage (control)	6.10 ± 0.04	0.02 ± 0.01	0.12 ± 0.06
	30	4.48 ± 0.04[a]	0.13 ± 0.01[a]	1.14 ± 0.14[a]
	60	2.49 ± 0.02[a]	6.59 ± 0.47[a]	32.41 ± 0.99[a]

The solutions contained 30 mg/ml HCO-60 or PLS-20, shaken in a water bath under fluorescent light of about 500 lux for 20 d. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *a)* $p<0.01$.

incubation, three peaks of MCZ degradation products were observed, and the absorbances of these products were greater at 254 than 272 nm. In the HCO-60 solution without MCZ, these peaks were not observed under the same conditions. Table II shows the pH, FA concentration, residual amount of MCZ and ratios of the degradation products to MCZ after incubation at various temperatures for 8 d. No changes in the pH and FA concentration were observed at 30 °C, but changes occurred at temperature above 40 °C, indicating increased degradation of HCO-60. MCZ degradation was not observed at 30 °C, but degradation products were detected at 40 °C or above. No changes were observed when the air was replaced with nitrogen, indicating that the degradation of HCO-60 and MCZ is an oxygen-dependent oxidative reaction. These results indicate that MCZ degradation occurs with the degradation of HCO-60.

The effects of D-HCO-60 on MCZ degradation were examined after 16 d of storage at 30 °C. As shown in Table III, the addition of D-HCO-60 increased the degradation of MCZ.

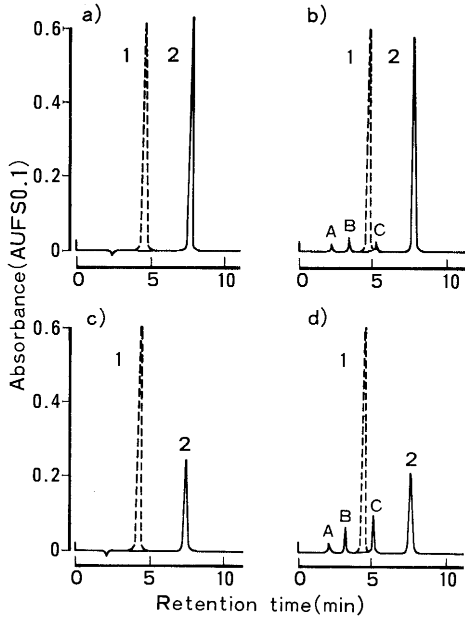

Fig. 2. Chromatograms of MCZ in the Solution Containing HCO-60

a, b) 272 nm; c, d) 254 nm; a, c) before storage; b,d) after 8 d of storage at 60 °C. Peak 1, internal standard; peak 2, MCZ. The solutions contained 10 mg/ml MCZ, 100 mg/ml HCO-60, 1 mg/ml lactic acid in a final volume of 1 ml, sealed in ampoules (inner volume: 3.5 ml).

Table II. Stability of the MCZ Solutions Containing HCO-60 at Various Temperatures

Storage temperature (°C)	pH	FA concentration (mM)	Residual amount of MCZ (%)	Ratio[a] of degradation product C to MCZ
Before storage (control)	4.40 ± 0.01	0.15 ± 0.02	100.0 ± 0.8	0
30	4.44 ± 0.03	0.19 ± 0.02	99.8 ± 0.7	0
40	4.20 ± 0.02[d]	2.06 ± 0.36[d]	95.2 ± 1.4[d]	0.39 ± 0.05
50	4.02 ± 0.02[d]	3.29 ± 0.07[d]	96.5 ± 1.3[c]	0.37 ± 0.01
60	3.88 ± 0.01[d]	3.77 ± 0.16[d]	94.4 ± 1.4[d]	0.33 ± 0.02
60, N_2[b]	4.4 ± 0.01	0.16 ± 0.02	99.5 ± 0.6	0

The solutions contained 10 mg/ml MCZ, 100 mg/ml HCO-60 and 1 mg/ml lactic acid in a final volume of 1 ml, sealed in ampoules (inner volume: 3.5 ml) and stored for 8 d. *a)* Peak area of degradation product C in Fig. 1/peak area of MCZ, at 254 nm. *b)* Replaced with nitrogen gas. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *c)* $p<0.05$, *d)* $p<0.01$.

Table III. The Effect of D-HCO-60 on Stability of MCZ Solutions

HCO-60 added	pH	Change of FA concentration (mM)	Residual amount of MCZ (%)	Ratio of degradation product C to MCZ
F-HCO-60[a] (control)	4.46 ± 0.02	0.21 ± 0.02	101.4 ± 0.6	0
F-HCO-60 + D-HCO-60[b]	4.03 ± 0.05[c]	1.93 ± 0.32[c]	94.2 ± 0.8[c]	0.50 ± 0.11

The solutions contained 5 mg/ml MCZ, 65 mg/ml HCO-60 and 0.5 mg/ml lactic acid in a final volume of 1 ml, sealed in ampoules (inner volume: 3.5 ml) and stored at 30°C for 16 d. *a)* Freshly prepared. *b)* D-HCO-60 concentration: 15 mg/ml. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *c)* $p<0.01$.

Table IV. The Effects of Various Substances on Stability of MCZ Solutions Containing HCO-60

Substances added	pH	Change of FA concentration (mM)	Residual amount of MCZ (%)	Ratio of degradation product C to MCZ
None (control)	4.34 ± 0.03	0.43 ± 0.10	96.8 ± 1.9	0.35 ± 0.09
FA[a]	4.38 ± 0.02	0.37 ± 0.13	101.1 ± 1.7[d]	0.16 ± 0.04[d]
Fe(II)[b]	4.28 ± 0.03	0.56 ± 0.01	97.1 ± 1.3	0.29 ± 0.01
H_2O_2[c]	4.28 ± 0.03	0.65 ± 0.11	92.4 ± 2.7	0.55 ± 0.09
H_2O_2[c] + Fe(II)[b]	3.93 ± 0.05[e]	1.01 ± 0.03[e]	82.1 ± 0.4[e]	0.73 ± 0.12[d]

The solutions contained 5 mg/ml MCZ, 50 mg/ml HCO-60, 0.5 mg/ml lactic acid and various substances in a final volume of 1 ml, sealed in ampoules (inner volume: 9 ml) and stored at 30°C for 8 d. *a, c)* 5 mM; *b)* 0.5 mM $FeSO_4$. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *d)* $p<0.05$, *e)* $p<0.01$.

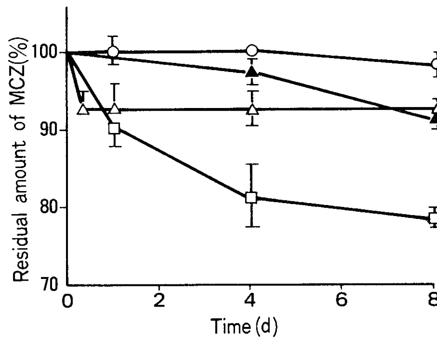

Fig. 3. The Effects of HCO-60 and Hydroxy Radicals on MCZ Degradation in 2 M Acetic Acid

The 2 M acetic acid solutions containing 10 mg/ml MCZ, 5 mM H_2O_2 and 0.5 mM $FeSO_4$ (△, ▲), 100 mg/ml HCO-60 (□), none (○) in a final volume of 1 ml were sealed in ampoules (inner volume: 9 ml) and stored for 8 d. Open symbols, at 60 °C; closed symbols, at 20 °C. Each value represents the mean ± S.D. of 3 experiments.

The Effects of FA, Fe(II), and H_2O_2 on Stability of MCZ Solutions Containing HCO-60 The stability of MCZ solutions stored for 16 d at 30 °C was studied in the presence of FA, Fe(II), H_2O_2 and both Fe(II) with H_2O_2 (Table IV). FA inhibited the degradation of HCO-60 and MCZ. Fe(II), H_2O_2 alone had no marked effect on HCO-60 or MCZ degradation. However, both Fe(II) with H_2O_2 increased the degradation of HCO-60 and MCZ.

The Effects of HCO-60 and Hydroxy Radicals on MCZ Degradation in 2 M Acetic Acid The effects of HCO-60 and hydroxy radicals on MCZ were studied using 2 M acetic acid, which can solubilize MCZ without HCO-60 (Fig. 3). Similar to the aqueous solution, MCZ was degraded by the addition of HCO-60. The generation of hydroxy radicals by a mixture of H_2O_2 and Fe(II) is known as the Fenton reaction.[3] The residual amount decreased to 92.6% after only 8 h of incubation at 60 °C, at which time H_2O_2 was completely consumed. Therefore, it was thought that little change in the residual amount after 8 h occurred because of the termination of the Fenton reaction. At 20 °C, the residual amount decreased gradually to 91.8% after 8 d of incubation, when the remaining amount of H_2O_2 was more than 90%. These results suggest the participation of hydroxy radicals in MCZ degradation.

The Effects of Hydroxy Radical Scavengers on MCZ Degradation in Solutions Containing HCO-60 Hydroxy radical scavengers were added to examine whether they prevent MCZ degradation in the solutions of HCO-60 (Table V). Thiourea and KI completely prevented degradation after 8 d of incubation at 60 °C, causing the action of cysteine, trolox and butanol to become weaker in that order.

Table V. The Effects of Hydroxy Radical Scavengers on MCZ Degradation in the Solution Containing HCO-60

Substance added	Residual amount of MCZ (%)
None (control)	77.0 ± 0.2
KI	100.6 ± 1.6[a]
Thiourea	100.5 ± 1.2[a]
Cysteine	92.8 ± 3.4[a]
Trolox	87.1 ± 0.7[a]
Butanol	80.3 ± 1.0[a]
Dimethylsulfoxide	77.7 ± 1.3

The solutions contained 5 mg/ml MCZ, 50 mg/ml HCO-60, 0.5 mg/ml lactic acid and 25 mM scavengers in a final volume of 1 ml, sealed in ampoules (inner volume: 9 ml) and stored at 60 °C for 8 d. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *a)* $p<0.01$.

Table VI. The Effects of LAR-9, PLS-20 and PEG-400 on MCZ Degradation

Substances added	Residual amount of MCZ (%)	Ratio of degradation product C to MCZ
None (control)	98.3 ± 1.6	0.25 ± 0.02
LAR-9	75.7 ± 0.3[a]	0.73 ± 0.01[a]
PLS-20	79.3 ± 0.7[a]	1.05 ± 0.05[a]
PEG-400	93.0 ± 0.3[a]	0.82 ± 0.03[a]

The 2 M acetic acid solutions containing 10 mg/ml MCZ and 100 mg/ml various polyoxyethylene surfactants in a final volume of 1 ml were sealed in ampoules (inner volume: 9 ml) and stored for 8 d. Each value represents the mean ± S.D. of 3 experiments. Significantly different from the control, *a)* $p<0.01$.

The Effects of Other Polyoxyethylene Surfactants on MCZ Degradation Table VI shows the residual amount of MCZ and the ratio of degradation product C to MCZ in 2 M acetic acid after the addition of LAR-9, PLS-20, and PEG-400. All of these surfactants increased MCZ degradation.

Discussion

Autoxidation of lipids containing unsaturated fatty acids has been extensively investigated, but the EO chain is known to be autoxidized in a manner similar to lipids.[1] In our experiment, autoxidation of HCO-60 and PLS-20 produced HPO, FA and formic acid (Fig. 1, Table I). This result is consistent with the previous report that HPO was formed in the EO chain of polyoxyethylene surfactants and that the degradation of HPO resulted in aldehydes and acids.[1] There have been some investigations on the effects of this process on drugs,[4] but the agent that produces these effects has not been identified. We found that the degradation of MCZ was associated with that of HCO-60 and was an oxygen-dependent process (Table II). The degradation of MCZ was not temperature dependent. Its cause remains obscure. The degradation of MCZ was accelerated by the addition of D-HCO-60 (Table III). These findings indicate that degradation of HCO-60 induces degradation of MCZ. The initiation period of this chain reaction is known to be considerably shortened, and the reaction speed to be

increased, by the addition of D-HCO-60 at the beginning of autoxidation.[1a)] We considered this to have increased the degradation of MCZ. We have demonstrated that this acceleration of degradation occurs not only for MCZ but also for doxycycline hydrochloride and adriamycin hydrochloride.[5)] Since these drugs are generally less stable and more readily oxidized than MCZ, the effects of the addition of D-HCO-60 were greater than in MCZ.

To more closely evaluate the mechanism of this degradation, the effects of the addition of FA, Fe(II), and H_2O_2 and of Fe(II) and H_2O_2 on HCO-60-containing MCZ solutions were examined (Table IV). The residual amount of MCZ decreased at 30 °C even without these agents, and degradation products were detected. Although the results did not agree with those shown in Table II, this may be ascribed to differences in the HCO-60 concentration and the head space of the ampoules.[1a)] As degradation of HCO-60 and MCZ are suppressed by the addition of FA, which is a degradation product of HCO-60, an agent that degrades MCZ is produced in the oxidation process before the generation of FA. Fe(II) and H_2O_2 are promoters of oxidation of HCO-60, because the former catalyzes redox degradation, and the latter shortens the initiation period.[1a)] When Fe(II) or H_2O_2 were added alone, they had little effect on HCO-60 or MCZ in the present study. However, degradation of both HCO-60 and MCZ was increased when Fe(II) and H_2O_2 were added simultaneously. These results may be explained by the following reaction process.

The degradation process of HPO produced in EO chains is important for the progression of autoxidation. The O–O bond dissociation energy in HPO is about 42 kcal/mol, and the O–O bond is more readily dissociated than the C–H bond.[6)] Therefore, hydroxyradicals may be generated.

$$-(OCH_2\underset{\displaystyle OOH}{\underset{|}{C}}H)O- \rightarrow -(OCH_2\underset{\displaystyle O\cdot}{\underset{|}{C}}H)O- + \cdot OH$$

This chain branching caused the reaction to shift from the initiation period to the propagation period and exponentially accelerates the oxidative reaction. The hydroxy radicals produced in this process are highly active and may trigger the hydrogen-extraction reaction observed in the initiation period of autoxidation to produce alkyl radicals.[7)]

$$-(OCH_2CH_2)O- + \cdot OH \rightarrow -(OCH_2\dot{C}H)O- + H_2O$$

This may be a factor in promotion of the chain reaction. Therefore, degradation of HCO-60 was accelerated by the addition of hydroxy radicals. Hydroxy radicals derived from the Fenton reaction and degradation of HPO were responsible for degradation of MCZ. This was confirmed by experiments using acetic acid solution (Fig. 3). When hydroxy radicals were generated in solutions without HCO-60, the residual amount of MCZ decreased to 92.6% after incubation at 60 °C for 8 h. No further changes in the residual amount were observed, because H_2O_2 had mostly been degraded and no additional hydroxy radicals were generated. Degradation gradually proceeded even at 20 °C, and the residual amount after 8 d decreased to 91.8%. These findings suggest that hydroxy radicals degrade MCZ. The inhibitory effects of hydroxy radical scavengers on MCZ degradation were studied next (Table V). The effect of each hydroxy radical scavenger was consistent with the reports of Ozawa,[7b)] Ishimitsu *et al.*[8)] and Nishida *et al.*[9)]

The above observations indicate that hydroxy radicals generated by autoxidation of HCO-60 are mainly responsible for the degradation of MCZ. It was confirmed that degradation of MCZ is also induced by other polyoxyethylene surfactants (Table IV). This finding may explain the above-mentioned degradation process. During degradation of MCZ, not only hydroxy radical but also some other radicals (alkoxy radical, peroxy radical, *etc.*) are probably formed. It is, however, unknown how these radicals are involved in the degradation of MCZ. Hydroxy radicals are highly reactive and toxic among active oxygen species.[7)] Anaphylactic shock has been occasionally reported after intravenous injection of surfactants containing EO chains,[10)] but the cause has not been clarified. Therefore, the relationship among hydroxy radicals resulting from autoxidation of EO chains, degradation of drugs, and shock remains to be elucidated.

Acknowledgements We are grateful to Nikko Chemicals Co., Ltd. and Mochida Pharmaceutical Co., Ltd. for the kind supply of HCO-60 and MCZ, respectively.

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Effect of Ion Species and Their Concentration on the Iontophoretic Transport of Benzoic Acid through Poly(vinyl acetate) Membrane

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Received April 10, 1991

Effects of ion species and their concentration on the iontophoretic transport of benzoic acid through an artificial membrane [poly(vinyl acetate)] were investigated using a 2-chamber diffusion cell equipped with platinum electrodes and a constant current power source. The cathode side of the cell was filled with sodium benzoate solution, and the anode side with potassium chloride, lithium chloride or tetraethylammonium bromide solution. When the molar concentration of sodium benzoate in the cathode side (0.21 M) was the same as the potassium chloride in the anode side, the amounts of benzoate anion and potassium cation permeated through the membrane were greater with increasing current. With an increase in the concentration of benzoate anion in the cathode side and a constant concentration of potassium cation in the anode side, the amount of the former that permeated was proportional to the concentration applied, while permeation of potassium cation remained almost constant at a constant current of 0.2 mA. Conversely, an increase in the concentration of potassium cation in the anode side with a constant concentration of benzoate anion in the cathode side resulted in an increase of the permeation of potassium cation and a decrease of that of benzoate anion, respectively, at the same constant current. When potassium chloride in the anode side was replaced by tetraethylammonium bromide, the amount of benzoate anion permeated was increased at a constant current of 0.2 mA. These results may be explained by the following theory on the transport number of each ion through the artificial membrane: Current density calculated from ion flux through the membrane was almost the same as that measured by observed current density. The results appeared to indicate that not only an ion species and its concentration in the donor solution but also ion species in the receiver solution should be considered when evaluating the iontophoretic transport phenomena.

Keywords iontophoresis; permeability; poly(vinyl acetate) membrane; transport number; sodium benzoate

Potent ionizable drugs such as peptides and proteins can now be easily synthesized by biotechnology, and it is important that a suitable dosage form and delivery route are designed for each drug. Peptide and protein drugs are highly susceptible to degradation in the gastrointestinal environment and hepatic "first-pass" metabolism when taken orally. Frequent injections are needed because of their short biological half life when taken parenterally. Transdermal administration is thus interesting to avoid "first-pass" metabolism and obtain typical sustained absorption by this administration route. The permeation rate of such drugs, however, is generally so poor that only low bioavailability is obtained through the skin. This poor permeability can be overcome by the use of one of several methods which include chemical enhancers and the application of external energy. Ultrasound or electric current is used as external energy to enhance the percutaneous absorption of several drugs. The two general categories of energy used are phonophoresis and iontophoresis. Iontophoresis can be defined as a process or method in which the permeation rate of ionic species into the body is enhanced by applying an electric current between viable tissues. Many studies have confirmed its usefulness in chemotherapy[1] and diagnostics,[2] and recent efforts have concentrated on understanding and elucidating the mechanism involved.[3] Few studies have been reported, however, on power sources, electrodes, composition of vehicles or pharmaceutical additives used with these iontophoretic systems.[4] The purpose of the present study was to gain basic information on the effect of ion species and ionic concentration on the iontophoretic transport of benzoic acid as a model drug using a 2-chamber diffusion cell. Poly(vinyl acetate) membrane was used as an artificial membrane to prevent the effect of biological ions on the transport phenomenon of the drug. The poly(vinyl acetate) membrane is useful because of its adequate permeation property against ions, adequate hydrophilicity (or lipophilicity) and comparative rigidity.

Experimental

Materials Sodium benzoate (Japanese Pharmacopeia grade) was obtained from Yamada Pharmaceutical Co., Ltd. (Ibaraki, Japan). Poly(vinyl acetate) (monomer unit = 1400—1600) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cold and radiolabelled tetraethylammonium bromide ([1-^{14}C]-, specific activity; 0.15 GBq/mmol, purity; 99%) were obtained from Wako Pure Chemical Industries, Ltd. and Du Pont New England Nuclear (Boston, MA, U.S.A.), respectively. 2,5-Diphenyloxazole was obtained from Wako Pure Chemical Industries, Ltd. and *p*-bis[2-phenyloxazolyl)]-benzene was obtained from Du Pont New England Nuclear to make a scintillation cocktail. Platinum wire (99.9% purity, 1 cm × 1 mm) was obtained from Tokuriki (Tokyo, Japan) for the electrodes. Other chemicals were of reagent grade. All reagents were used without further purification and all solutions were made with deionized water which had been passed through a water purifier (Eyela ER, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The resulting water had a pH of 6.74 and an electric conductivity of 0.39 μS cm^{-1}. Silicone-coated poly(ethylene terephthalate) (PET) film was a gift from Nichiban Co., Ltd. (Tokyo, Japan).

Membrane Preparation Poly(vinyl acetate) (3.7 g) was dissolved in ethyl acetate to get a 37% poly(vinyl acetate) solution. A sheet of silicone coated PET film (30 cm × 25 cm × 25 μm) was placed on a flat glass plate (with silicone-coated side up) and secured in place with silicone tape. The polymer solution (*ca.* 4 g) was poured carefully onto the silicone-coated PET film. An applicator (Ueshima, Tokyo, Japan) was then gently passed through the silicone-coated PET film to produce a 5 ± 1 μm thick membrane. The solvent (ethyl acetate) was allowed to evaporate for 3 min under 130 °C in a laboratory dryer. The resulting poly(vinyl acetate) membrane was measured by a dial thickness gauge (limit, 0.1 μm), and membrane with a thickness of 5 ± 1 μm was used in the permeation study.

Membrane Permeation Study A sheet of poly(vinyl acetate) membrane

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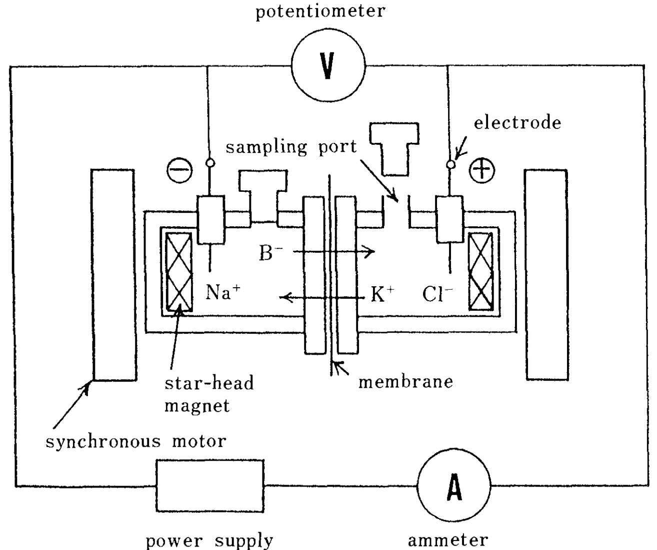

Fig. 1. Schematic Diagram of Ion Permeation Study

B^- means benzoate anion: the same as below.

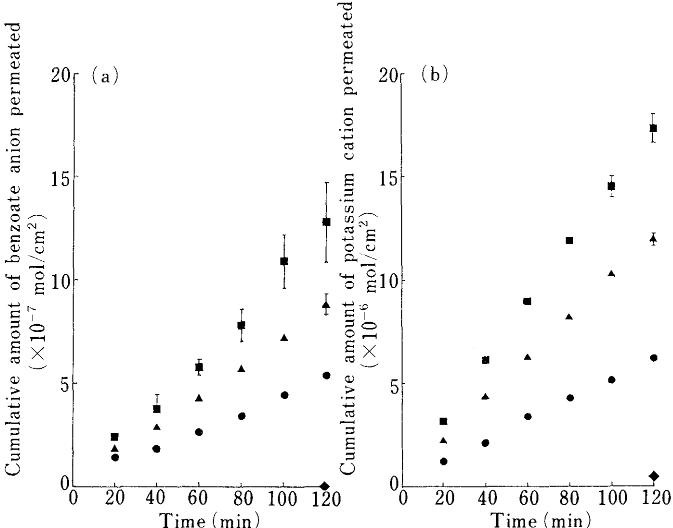

Fig. 2. Effect of Applied Current on the Benzoate Anion (a) and Potassium Cation (b) Permeated through the Poly(vinyl acetate) Membrane

Control; ◆, 0.1 mA; ●, 0.2 mA; ▲, 0.3 mA; ■. Cathode and anode sides are filled with 0.21 M sodium benzoate and potassium chloride solution, respectively. Each point represents the mean ± S.E. of three experiments.

was mounted between the cathode and anode sides of a 2-chamber diffusion cell which was equipped with platinum electrodes (Fig. 1). The surface area of the membrane available for ion permeation was 0.95 cm^2. A pair of platinum electrodes was immersed in the solutions in both sides of the cell. These electrodes were connected to a constant current power source (DMR-20-2, Metronix, Tokyo, Japan). Current was measured with a digital multimeter (TR6843, Takeda Riken, Tokyo, Japan) in series with the 2-chamber diffusion cell and constant current power source. The cathode side of the cell was filled with 4 ml of sodium benzoate solution (0.069, 0.21 or 0.35 M) and the anode side of the cell with the same volume of potassium chloride, lithium chloride or tetraethylammonium bromide solution (0.021, 0.21 or 2.1 M). A sample of 0.05 ml was withdrawn at predetermined intervals from either side of the cell, and the amount of ions permeated through the poly(vinyl acetate) membrane was determined.

Determination of Permeated Ion A high performance liquid chromatography system consisting of a pump (LC-6A, Shimadzu, Kyoto, Japan), an ultraviolet detector (SPD-6A, Shimadzu) and an integrator (C-R6A, Shimadzu) was used for analyzing benzoate anion concentration. Acetonitrile: 0.05 M phosphate buffer solution (pH 2.5) (1 : 1, v/v) was used as a mobile phase at a flow rate of 1.0 ml min^{-1}. Benzoate anion was resolved using a 4.6 x 250 mm stainless-steel column packed with Nucleosil 5C$_{18}$ (Macherey Nagel, Germany) and detected at a wavelength of 230 nm. Ten μg ml^{-1} of *p*-ethylbenzoate in methanol solution was used as an internal standard. A clinical ion meter (CIM-104A, Shimadzu) was used for analyzing potassium cation, and a flame emission spectrophotometer (detector AA-200, gas control unit JAG-5D, Nippon Jarrell-Ash Co., Ltd., Kyoto, Japan) to analyze lithium cation. Analytical conditions were as follows: Peak height method; oxidant, air 6.5 l min^{-1} at 2.45 kg cm^{-2}; fuel, C_2H_2 1.25 l min^{-1} at 0.5 kg cm^{-2}; wavelength, 670.5 nm. Cold tetraethylammonium bromide solution was spiked with hot (185 kBq) tetraethylammonium bromide solution in the anode side of the cell. A 0.05 ml sample was withdrawn at predetermined intervals from the cathode side and poured into vials containing the scintillation cocktail. Radioactivity was measured by a liquid scintillation counter (LSC-703, Aloka, Tokyo, Japan).

Results

Effect of Applied Current on Ion Permeation First, the cathode side was filled with 4 ml of 0.21 M sodium benzoate and the anode side was filled with the same volume of 0.21 M potassium chloride. Figure 2a shows the cumulative amount of benzoate anion permeated through the poly(vinyl acetate) membrane from the cathode side to the anode side, and Fig. 2b shows that of potasssium cation permeated through the membrane from the anode side to the cathode side.

Table I. Effect of Applied Current on the Enhancement Factor of Benzoate Anion and Potassium Cation[a)]

Applied current (mA)	Cumulative amount of ion permeated ($\times 10^{-7}$ mol/cm^2)		Enhancement factor[b)]	
	B^-	K^+	B^-	K^+
0	0.0537	5.05		
0.1	5.39	62.2	100.4	12.3
0.2	8.84	120.0	164.6	23.8
0.3	12.8	173.0	238.4	34.3

a) Cathode and anode sides were filled with 0.21 M sodium benzoate and potassium chloride solution, respectively.

b) Enhancement factor $= \frac{\text{cumulative amount of ion (iontophoresis)}}{\text{cumulative amount of ion (passive)}}$.

Table I shows the enhancement factor of the permeation of benzoate anion and potassium cation, which was calculated by dividing the cumulative amount of the two ions permeated in the first 2 h of the experiment by those of the control experiments (no applied current) (see legend of Table I). The permeations of benzoate anion and potassium cation were increased with an increase in the applied current.

Effect of Sodium Benzoate Concentration in the Cathode Side on Ion Permeation Next, the cathode side was filled with 4 ml of 0.069, 0.21 or 0.35 M sodium benzoate and the anode side with 4 ml of 0.21 M potassium chloride. Figure 3a shows the cumulative amount of benzoate anion permeated through the membrane from the cathode to the anode side, and Fig. 3b shows that of potassium cation permeated from the anode to the cathode side at a constant current of 0.2 mA. Table II shows the enhancement factor of the permeation of the two ions. When the concentration of benzoate anion in the cathode side was increased with the concentration of 0.21 M potassium cation remaining constant in the anode side, the amount of benzoate anion permeated was proportional to its applied concentration

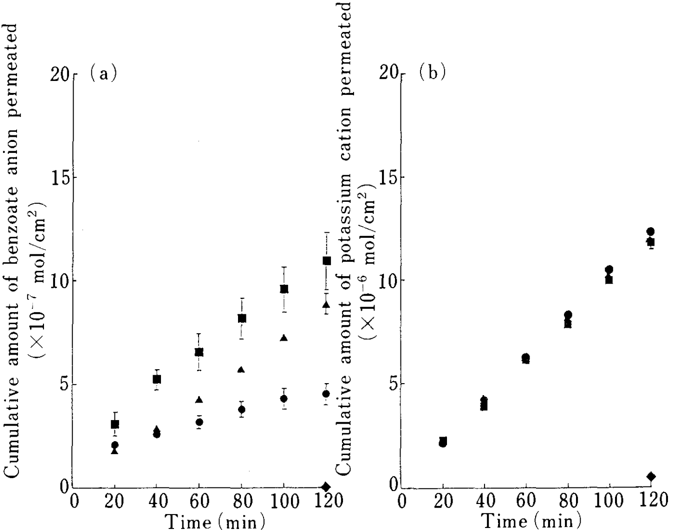

Fig. 3. Effect of Sodium Benzoate Concentration in the Cathode Side on the Benzoate Anion (a) and Potassium Cation (b) Permeated through the Poly(vinyl acetate) Membrane at a Constant Current of 0.2 mA

Control; ◆, 0.069 M; ●, 0.21 M; ▲, 0.35 M; ■. Anode side is filled with 0.21 M potassium chloride solution. Each point represents the mean ± S.E. of three experiments.

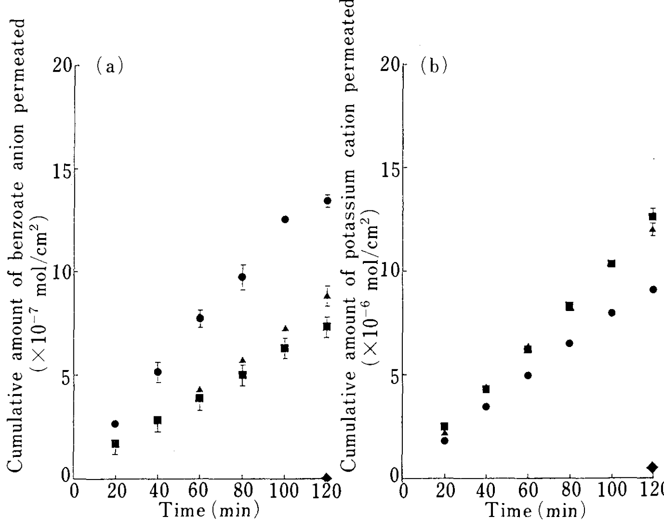

Fig. 4. Effect of Potassium Chloride Concentration in the Anode Side on the Benzoate Anion (a) and Potassium Cation (b) Permeated through the Poly(vinyl acetate) Membrane at a Constant Current of 0.2 mA

Control; ◆, 0.021 M; ●, 0.21 M; ▲, 2.1 M; ■. Cathode side is filled with 0.21 M sodium benzoate solution. Each point represents the mean ± S.E. of three experiments.

TABLE II. Effect of Ion Concentration of Cathode Side[a] on the Enhancement Factor of Benzoate Anion and Potassium Cation

Sodium benzoate conc. (M)	Cumulative amount of ion permeated (×10^{-7} mol/cm^2)		Enhancement factor[b]	
	B^-	K^+	B^-	K^+
Control	0.0537	5.05	—	—
0.069	4.49	123.0	83.6	24.4
0.21	8.84	120.0	164.6	23.8
0.35	10.9	118.0	203.0	23.4

a) Anode side was filled with 0.21 M potassium chloride solution. b) See the legend of Table I.

TABLE III. Effect of Ion Concentration of Anode Side[a] on the Enhancement Factor of Benzoate Anion and Potassium Cation

Potassium chloride conc. (M)	Cumulative amount of ion permeated (×10^{-7} mol/cm^2)		Enhancement factor[b]	
	B^-	K^+	B^-	K^+
Control	0.0537	5.05	—	—
0.021	13.3	90.2	247.7	17.9
0.21	8.84	120.0	164.6	23.8
2.1	7.31	126.0	136.1	25.0

a) Anode side was filled with 0.21 M potassium chloride solution. b) See the legend of Table I.

and the amount of potassium cation permeated was almost constant at the constant current of 0.2 mA. Table II shows the enhancement factor of the two ions.

Effect of Potassium Chloride Concentration in the Anode Side on Ion Permeation The cathode side was then filled with 4 ml of 0.21 M sodium benzoate and the anode side with 4 ml of 0.021, 0.21 or 2.1 M potassium chloride. Figure 4a shows the cumulative amount of benzoate anion permeated through the membrane from the cathode to the anode side, and Fig. 4b shows that of potassium cation permeated from the anode to the cathode side at a constant current of 0.2 mA. Table III shows the enhancement factor for the two ion permeations. When the concentration of potassium cation in the anode side was increased while retaining a constant concentration of benzoate anion in the cathode side, the permeating amounts of these two ions were respectively increased and decreased.

Effect of Ion Species in the Anode Side on the Ion Permeation The cathode side was next filled with 4 ml of 0.21 M sodium benzoate and the anode side with 4 ml of 0.021 M potassium chloride, lithium chloride or tetraethylammonium bromide. Figure 5a shows the cumulative amount of benzoate anion permeated through the mem-

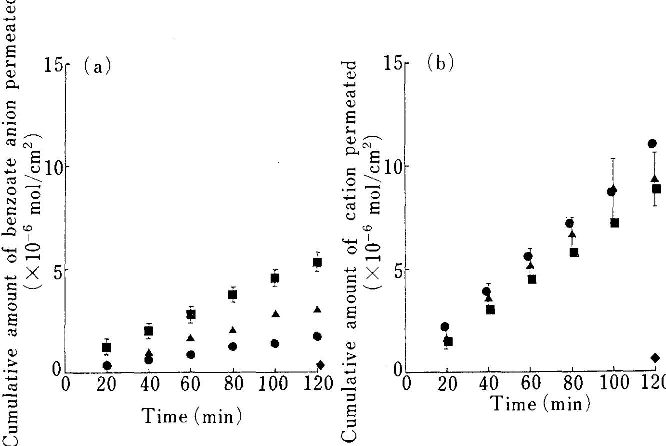

Fig. 5. Effect of Ion Mobility in the Anode Side on the Benzoate Anion (a) and Each Cation (b) Permeated through the Poly(vinyl acetate) Membrane at a Constant Current of 0.2 mA

◆, control; ●, potassium cation; ▲, lithium cation; ■, tetraethylammonium cation. Cathode side is filled with 0.21 M sodium benzoate solution and anode side is filled with 0.021 M potassium chloride, lithium chloride or tetraethylammonium bromide solution. Each point represents the mean ± S.E. of three experiments.

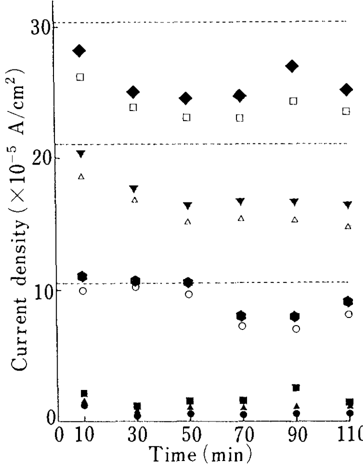

Fig. 6. Time Course of the Total Current Density Calculated from the Amount of Ion Permeated

●, 0.1 mA benzoate anion; ▲, 0.2 mA benzoate anion; ■, 0.3 mA benzoate anion; ○, 0.1 mA potassium cation; △, 0.2 mA potassium cation; □, 0.3 mA potassium cation; ⬢, total current density of 0.1 mA; ▼, total current density of 0.2 mA; ◆, total current density of 0.3 mA. Total current density = current density calculated from benzoate anion permeated + current density calculated from potassium cation permeated.

brane from the cathode to the anode side, and Fig. 5b shows that of potassium, lithium or tetraethylammonium cation permeated from the anode to the cathode side at a constant current of 0.2 mA. When the ion species in the anode side was changed from potassium cation to tetraethylammonium cation, the amount of benzoate anion permeated from the cathode to the anode side and that of the cation permeated from the anode to the cathode side were increased and decreased, respectively, at a constant current of 0.2 mA.

Time Course of Current Calculated from the Amount of Ions Permeated Figure 6 shows the time course of current density calculated from fluxes of benzoate anion from the cathode to the anode side and potassium cation from the anode side to the cathode side through the poly(vinyl acetate) membrane. The time course as calculated in this manner was almost the same as that measured by the observed current density. The difference in the current density calculated from ion flux and observed current density increased with the increase in applied current.

Discussion

In studying iontophoretic membrane permeation using a 2-chamber diffusion cell, not only drug ion (benzoate anion) transport but the transport of other ion species which contribute to the current through the membrane require consideration. Since fluxes of sodium cation from the cathode side to the anode side in the sodium benzoate solution and of chloride anion from the anode to the cathode side in the potassium chloride solution were almost negligible in the present study, the electric current through the poly(vinyl acetate) membrane obeys the following relationship:

$$I_{tot} = Z_{B^-} \times F \times C_{B^-} \times U_{B^-} \times E \times S + Z_{K^+} \times F \times C_{K^+} \times U_{K^+} \times E \times S \quad (1)$$

where I_{tot} is total current through the membrane, Z, F, C, U, E and S are, respectively, charge of ion, Faraday constant (96485 $C\,mol^{-1}$), concentration of ion ($mol\,cm^{-3}$), ionic mobility ($cm^2\,s^{-1}\,V^{-1}$), potential gradient ($V\,cm^{-1}$) and surface area of membrane available for ion permeation (cm^2).[5] Subscripts B^- and K^+ show benzoate and potassium ions, respectively. In general, flux of an ion i through membrane can be obtained using the following equation:

$$J_i = C_i \times U_i \times E \quad (2)$$

where J_i is flux of ion$_i$ ($mol\,cm^{-2}\,s^{-1}$). Using Eq. 3, Eq. 1 is transformed to:

$$I_{tot}/S = Z_{B^-} \times F \times J_{B^-} + Z_{K^+} \times F \times J_{K^+} \quad (3)$$

where I_{tot}/S is total current density and $Z_{B^-} \times F \times J_{B^-} + Z_{K^+} \times F \times J_{K^+}$ is current density due to the transport of benzoate anion from the cathode to the anode side and that of potassium cation from the anode to the cathode side. Total current density (left side of Eq. 3) can be determined by measuring the current in the iontophoretic membrane permeation experiment, and $\Sigma\, Z_i \times F \times J_i$ (right side of Eq. 3) can be calculated by fluxes of K^+ and B^-. Transport number of each ion species is defined as:

$$t_{B^-} = Z_{B^-} \times F \times J_{B^-} / (Z_{B^-} \times F \times J_{B^-} + Z_{K^+} \times F \times J_{K^+}) \quad (4)$$

$$t_{K^+} = Z_{K^+} \times F \times J_{K^+} / (Z_{B^-} \times F \times J_{B^-} + Z_{K^+} \times F \times J_{K^+}) \quad (5)$$

where t_{B^-} and t_{K^+} are the transport numbers of benzoate anion and potassium cation, respectively. The flux of each ion contributes to the transport number of that ion. Concentration of ion (C_i) and ionic mobility (U_i) also contributes to the transport number since they influence the ion flux as shown in Eq. 2.

The amount of benzoate anion and potassium cation permeated through the membrane was extremely low without current (passive diffusion alone) (Fig. 2). The current flow from one electrolyte through a membrane to another electrolyte is usually the result of ion transfer. As the applied current increased, the amounts of benzoate anion and potassium cation permeated through the membrane were increased (Fig. 2).

When the concentration of benzoate anion in the cathode side was increased, the amount of ion permeated was proportional to its concentration and the amount of potassium cation permeated was almost constant (Fig. 3). The ionic mobility of potassium cation was about 13 times greater than that of benzoate anion when the molar concentration of sodium benzoate in the cathode side was the same as that of potassium chloride in the anode side (Fig. 6). Table IV shows transport numbers of benzoate anion and potassium cation calculated by Eqs. 4 and 5. Figure 7 shows the relationship between sodium benzoate concentration in the cathode side and the transport number

Table IV. Effect of Sodium Benzoate Concentration in the Cathode Side on the Transport Number

Sodium benzoate conc. (M)	Transport number	
	B^-	K^+
0.069	0.025	0.975
0.21	0.071	0.929
0.35	0.114	0.886

Anode side was filled with 0.21 M potassium chloride solution.

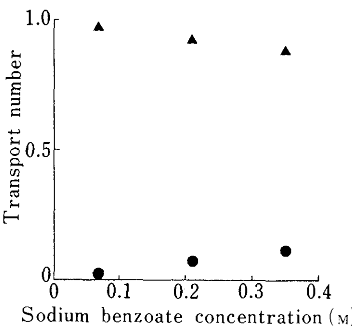

Fig. 7. Relationship between Sodium Benzoate Concentration in the Cathode Side and Transport Number of Benzoate Anion and Potassium Cation at a Constant Current of 0.2 mA

●, sodium benzoate; ▲, potassium chloride. Anode side is filled with 0.21 M potassium chloride solution.

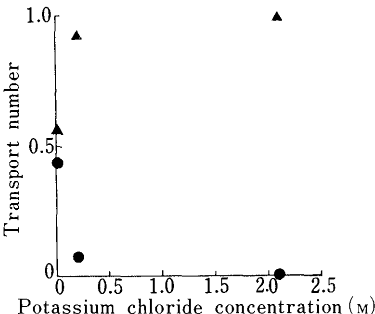

Fig. 8. Relationship between Potassium Chloride Concentration in the Anode Side and Transport Number of Benzoate Anion and Potassium Cation at a Constant Current of 0.2 mA

●, sodium benzoate; ▲, potassium chloride. Cathode side is filled with 0.21 M sodium benzoate solution.

Table V. Effect of Potassium Chloride Concentration in the Anode Side on the Transport Number

Potassium chloride conc. (M)	Transport number	
	B^-	K^+
0.021	0.435	0.565
0.21	0.071	0.929
2.1	0.008	0.992

Cathode side was filled with 0.21 M sodium benzoate solution.

of B^- and K^+. The amount of potassium cation permeated was not affected when the molar concentration of sodium benzoate in the cathode side was changed due to the much greater mobility of potassium cation than that of benzoate anion.

As the concentration of potassium cation in the anode side was increased, the amounts of benzoate anion and potassium cation permeated were decreased and increased, respectively (Fig. 4). Table V shows transport numbers of benzoate anion and potassium cation calculated by Eqs. 4 and 5. Figure 8 shows the relationship between potassium chloride concentration and transport number. The amount of benzoate anion permeated was increased when the molar concentration of potassium chloride in the anode side was decreased, because the ion mobility of potassium cation is greater than that of benzoate anion.

When potassium cation in the anode side was replaced by another cation such as lithium or tetraethylammonium cation, the amount of benzoate anion permeated from the cathode side to the anode side and the amount of cation permeated were increased and decreased, respectively (Fig. 5). It was thought that the amount of benzoate anion permeated was affected by the mobility of ions in the anode side. The ion mobility contributes also to the total current, and hence the transport number of benzoate anion was increased, especially when ion with low mobility (tetraethylammonium cation) was applied in the anode side.

The time course of current density calculated by Eq. 3 was almost the same as the observed current density (Fig. 6). The difference between the current density calculated by total ion flux (ion influx + efflux) and observed current density measured by an ammeter was greater with the increase in applied current. This difference may be due to a drop in voltage between the two platinum electrodes and an increase in electrolysis in both half cells with the increase in applied current.

Conclusion

The results of these studies suggested that not only the ion species and their concentration in the donor solution but also ion species in the receiver solution should be taken into consideration in any evaluation of the iontophoretic transport phenomenon. Clear understanding about the effects of ion species, ionic mobility and their concentration on the iontophoretic transport will lead to the design and discovery of well-assemblied iontophoretic devices.

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Solid Dispersions of Indomethacin and Griseofulvin in Non-porous Fumed Silicon Dioxide, Prepared by Melting[1]

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A method of increasing the dissolution rates of glass-forming, poorly water-soluble drugs was investigated. It is based on the concept of preparing the powder form of a glassy drug dispersed in an inert carrier by melting. Indomethacin (IMC) and griseofulvin were used as model drugs, and fumed silicon dioxide was used as the carrier. The drug–silica (1 : 2) mixture was heated until the drug melted. The properties of the solid dispersion thus obtained were examined using powder X-ray diffractometry (XRD), differential scanning calorimetry (DSC), infrared (IR) spectroscopy, and scanning electron microscopy (SEM). XRD showed that the drugs were converted from a crystalline state to an amorphous state in the solid dispersions. On DSC thermograms, a pure IMC glass showed an endothermic peak corresponding to glass transition, then an exothermic peak corresponding to transformation to a metastable crystal and an endothermic peak corresponding to melting of the metastable form. The IMC–silica solid dispersion, however, shows no transition peak in its thermogram, indicating that a solid dispersion is more stable to heat than pure glass. The drug–silica solid dispersions showed IR spectra characteristic of pure amorphous drugs but somewhat different from those of crystalline drugs. SEM revealed that the drug–silica solid dispersions had none of the characteristics observed in crystalline drugs. The dissolution rates of drugs from the solid dispersions were much higher than those from physical mixtures and from pure crystalline drugs.

Keywords indomethacin; griseofulvin; solid dispersion; amorphous; fumed silicon dioxide; dissolution rate

Introduction

In theory, a crystalline drug can be converted to the amorphous state *via* a gaseous, liquid, or crystalline solid state. Nakai *et al.* reported that the crystalline drugs interact with porous powders or controlled pore glasses, ultimately assuming the amorphous state.[2a–c] They proposed a mechanism whereby the drug molecules sublime and are adsorbed onto the surface of the pores. Konno reported that the vapor pressures of drugs correlate with the rate of transformation from the crystalline state to the amorphous state in the presence of an adsorbent.[2d] The above studies indicate the existence of the gas route. As a typical example of the liquid route, Borka reported that a brittle, glassy amorphous mass of indomethacin was obtained by cooling the melt.[3] Nakai *et al.* demonstrated that all crystalline drugs become amorphous on being ground with crystalline cellulose.[4a] Otsuka *et al.* also reported that indomethacin (IMC) was converted to the amorphous state on grinding with an agate centrifugal ball mill.[4b] Such crystal modifications on grinding may suggest the existence of a crystalline solid route as well as the other routes, but clarification of this possibility will require further study.

Some drugs have been shown to be able to form glass readily upon cooling from the liquid state. Although the glass has the potential to increase the dissolution rate, it is thermodynamically unstable and is therefore transformed to a stable crystalline state by processing and environmental stresses. Several investigators reported these phenomena: the glass tended to devitrify on grinding[5a–b]; powdered glass did not increase the dissolution rate because of its rapid devitrification on coming in contact with water[5c]; and powdered glass was converted to crystal on aging much faster than untreated glass.[5d–e] These results indicate that pure glass is not suitable for use in formulations.

One possible approach to solving the problems caused by the grinding of the glass is to prepare the powder form of the glass dispersed in an inert carrier. In the present study, we prepared solid dispersions of this type by melting, using IMC and griseofulvin (GRIS) as glass-forming, poorly water-soluble drugs, and non-porous fumed silicon dioxide (silica 380) as the carrier. This carrier is a pharmaceutical excipient consisting of amorphous, water-insoluble, hydrophilic, ultra-fine, spherical particles with extensive surface areas ($380\,m^2/g$). The purpose of this work was to evaluate this new approach to preparing glass powder by examining the properties of the solid dispersions using X-ray diffractometry (XRD), differential scanning calorimetry (DSC), infrared (IR), scanning electron microscopy (SEM) and dissolution testing.

Experimental

Materials Indomethacin U.S.P. grade (form I[3]) and griseofulvin (Nakarai Chemical Co., Ltd.), and non-porous fumed silicon dioxide (aerosil 380, Nippon Aerosil Co., Ltd.) were used without further purification.

Preparation of Physical Mixtures The drug and silicon dioxide were weighed accurately in various proportions, mixed for 3 min with a Vortex mixer, and were then passed through a 200-mesh sieve.

Preparation of Triturated Mixtures The drug and silicon dioxide were manually triturated with a pestle for 5 min in an agate mortar, and were passed through a 200-mesh sieve.

Preparation of Solid Dispersion Preliminary experiments showed that the factors involved in the preparation of solid dispersion by melting are drug–silicon dioxide ratios, heating temperature and mixing methods. Since these factors are interrelated, the drug–carrier ratios required for preparation varied depending on the temperature and mixing methods used. In this study, the following conditions were employed: triturated mixtures of IMC or GRIS with silicon dioxide (1 : 2, w/w) were transferred to an open petri dish and heated at 150 °C for 30 min and at 215 °C for 30 min, respectively; they were then allowed to cool to room temperature.

Differential Scanning Calorimetry (DSC) DSC thermograms were obtained using a Perkin–Elmer DSC-1B calorimeter. The samples were placed in aluminum sealed pans, and analyzed by heating at a rate of 16 °C/min in nitrogen gas. Temperature calibrations were made using stearic acid (69.4 °C), benzoic acid (122.4 °C), indium (155.6 °C), tin (231.9 °C), and lead (327.4 °C) as standards. The heats of fusion were calculated by gravimetrically measuring the peak areas of the sample and the indium standard.

X-Ray Diffraction Studies Powder X-ray diffraction patterns were

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recorded using a Rigaku Denki Geiger Flex 2012 diffractometer with nickel-filtered CuK_α radiation. Voltage 35 kV, current 20 mA, receiving slit 0.15 mm, scanning speed 2°/min, chart speed 20 mm/min.

IR Spectroscopy IR spectra were recorded using KBr disks on a Hitachi 270-30 infrared spectrophotometer.

Scanning Electron Microscopy Electron micrographs were made using a scanning electron microscope (JEOL JSM-T120). The samples were coated with gold, using a direct current sputter technique.

Dissolution Studies Dissolution testing was conducted using the JP XI paddle method. The dissolution medium was 900 ml of JP XI 1st fluid (pH 1.2) at 37 °C, the rotational speed of the paddle was 100 rpm, and the amount of sample was equivalent to 20 mg of the drug. At suitable time intervals, 5 ml samples of the dissolution fluid were withdrawn through a membrane filter (type HA, 0.45 μm, Millipore) and replaced with an equal volume of the dissolution fluid. The filtrates were suitably diluted and assayed spectrophotometrically at 252 nm for IMC and 260 nm for GRIS.

Results and Discussion

Figure 1 shows the powder X-ray diffraction patterns for the IMC–silica 380 (1 : 2) solid dispersion, physical and triturated mixtures and pure IMC crystal. The solid dispersion showed a halo pattern, and an absence of IMC diffraction peaks, indicating the presence of IMC in an amorphous form or as an extremely fine crystallite. Physical or triturated mixtures, on the other hand, showed diffraction peaks corresponding to form I of the IMC crystal. Similar results were obtained for the GRIS–silica 380 (1 : 2) system, as shown in Fig. 2.

When the IMC–silica 380 (1 : 2) mixtures were heated at 150 °C for 30 min, the physical mixture still showed diffraction peaks of crystalline IMC. In contrast, the triturated mixture lost diffraction peaks, as shown in Fig. 1. At 156 °C, the physical mixture required 30 min for conversion to the amorphous state, but the triturated mixture took only 20 min. To clarify the differences in solid-state properties between the physical and triturated mixtures, thermal analysis was performed using DSC.

The melting point (endothermic peak maximum) and heat of fusion (endothermic transition energy) obtained from DSC curves of IMC–silica 380 triturated mixtures decreased as the relative amount of silica 380 in the mixtures increased, as shown in Fig. 3. The lowering of these values was accompanied with broadening of the endothermic peaks corresponding to the melting of IMC. In the triturated mixture, the values fell much further than those of the physical mixture. On the other hand, a slight decrease in melting point and heat of fusion was also observed for the physical mixture containing 20% silica 380, but no further decrease in these values was observed even when the amount of silica 380 was increased.

The lower melting point of the IMC in the triturated

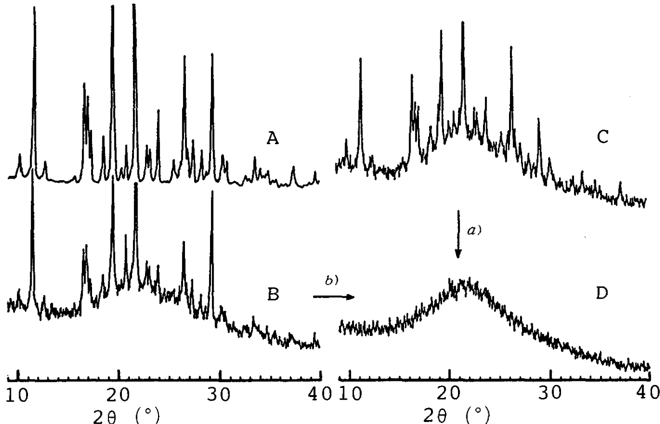

Fig. 1. Powder X-Ray Diffraction Patterns of IMC in Various Forms

A, IMC form I; B, IMC–silica 380 (1 : 2) physical mixture; C, IMC–silica 380 (1 : 2) triturated mixture; D, IMC–silica 380 (1 : 2) solid dispersion. *a)* 150 °C, 30 min or 156 °C, 20 min; *b)* 156 °C, 30 min.

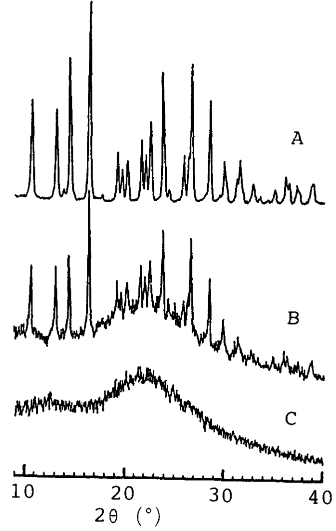

Fig. 2. Powder X-Ray Diffraction Patterns of GRIS in Various Forms

A, crystalline GRIS; B, GRIS–silica 380 (1 : 2) physical mixture; C, GRIS–silica 380 (1 : 2) solid dispersion.

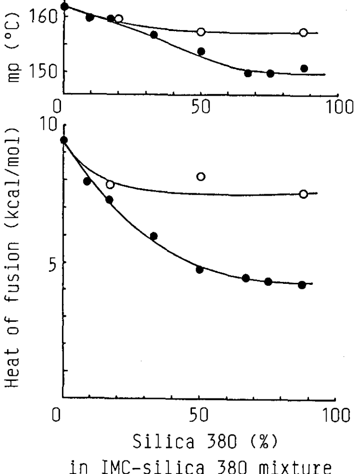

Fig. 3. The Peak Maximum and Energy of Endothermic Melting Transition of IMC–Silica 380 Mixtures as a Function of Composition

○, IMC–silica 380 physical mixture; ●, IMC–silica 380 triturated mixture.

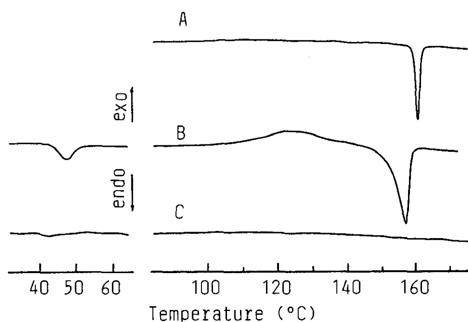

Fig. 4. Differential Scanning Thermograms of IMC in Various Forms

A, IMC form I; B, IMC glass; C, IMC–silica 380 (1 : 2) solid dispersion.

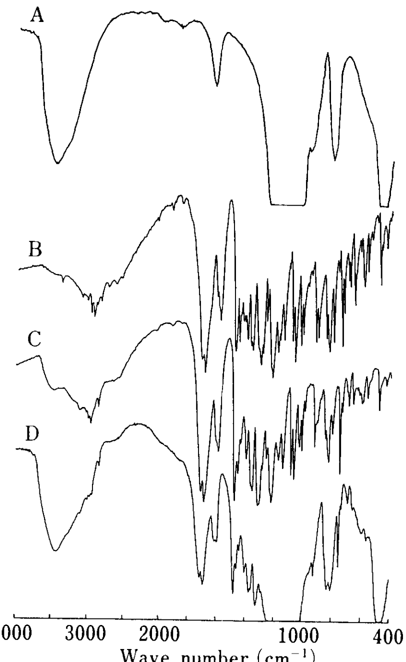

Fig. 5. IR Spectra of Silica 380 and IMC in Various Forms

A, silica 380; B, IMC form I; C, IMC glass; D, IMC–silica 380 (1 : 2) solid dispersion.

mixture explains why the IMC in this mixture was converted to the amorphous state at a lower temperature than IMC in the physical mixture. The lower heat of fusion explains why IMC in the triturated mixture was converted to the amorphous state faster than IMC in the physical mixture. As the trituration proceeded, the color of the triturated

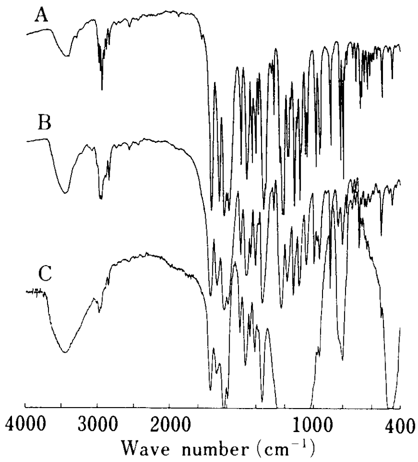

Fig. 6. IR Spectra of GRIS in Various Forms

A, crystalline GRIS; B, GRIS glass; C, GRIS–silica 380 (1 : 2) solid dispersion.

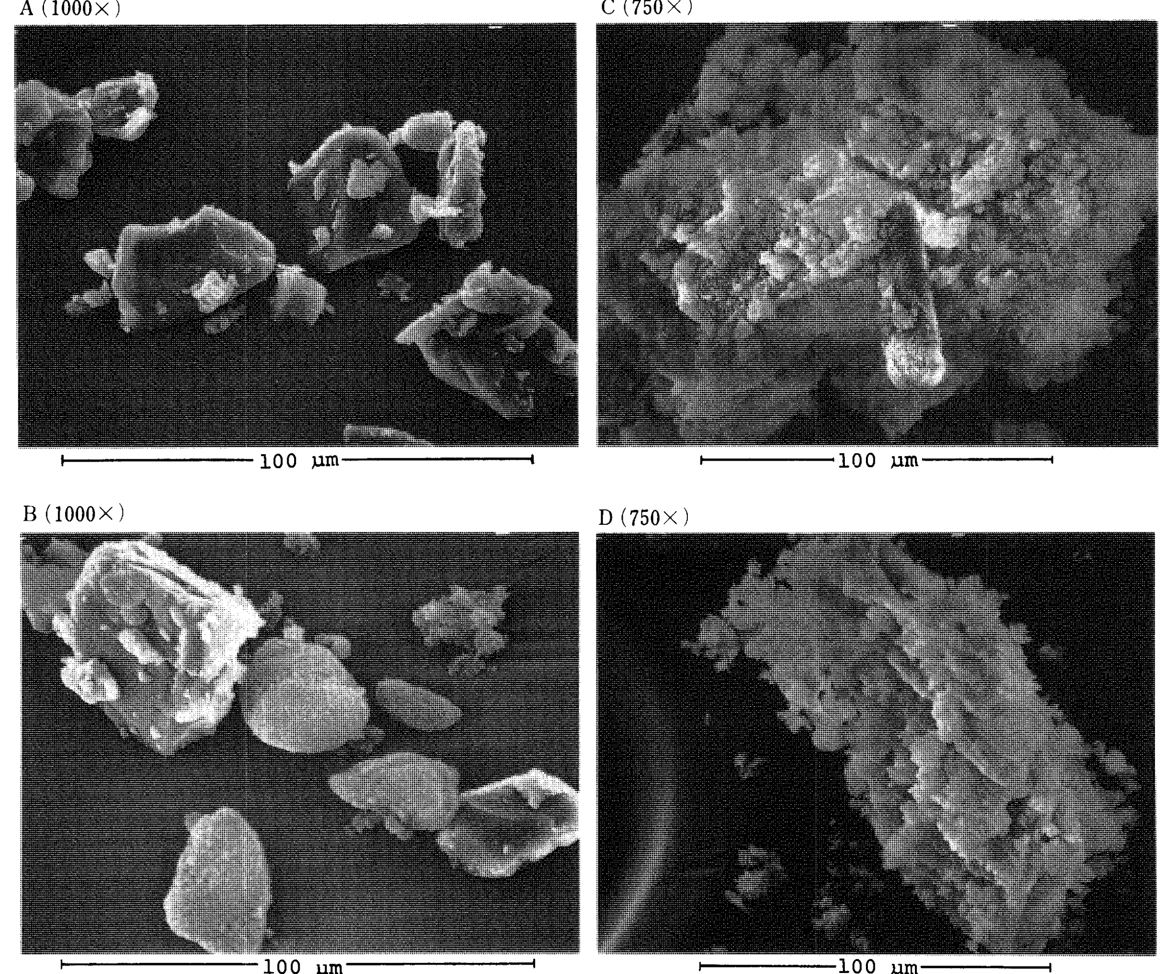

Fig. 7. Scanning Electron Micrographs of IMC in Various Forms

A, IMC form I; B, IMC–silica 380 (1 : 2) physical mixture; C, IMC–silica 380 (1 : 2) triturated mixture; D, IMC–silica 380 (1 : 2) solid dispersion.

sample changed from white to a slightly yellowish color that was characteristic of pure amorphous IMC glass, indicating that IMC becomes partly amorphous during trituration. It is likely that this crystal modification on trituration is caused by interactions between the IMC molecules and the silanol groups of the silica 380, resulting in the adsorption of some of the drug molecules onto the surface of the silica particles.[2c)]

Figure 4 shows DSC thermograms of pure crystalline IMC, IMC glass and an IMC–silica 380 (1 : 2) solid dispersion. Pure crystalline IMC showed an endothermic peak corresponding to the melting transition of form I. Pure IMC glass showed an endothermic peak due to glass transition at 44 °C, then an exothermic peak around 106 °C, and an endothermic peak at 153 °C, which corresponds to the melting point of form II. Borka reported that form II crystallized from the melt at 125—135 °C on a Kofler hot bench.[3)] Thus, the exothermic peak is due to crystallization of the amorphous form to the metastable form II. Solid dispersion, on the other hand, showed no change in its thermogram.

Silica 380 showed no transition peak over the temperature range examined, so that the thermogram of the solid dispersion should exhibit the thermal characteristics of IMC glass if no interaction occurs. The absence of a thermally detectable transition of a solid dispersion, therefore, indicates that the amorphous state of IMC is stabilized by the interaction associated with hydrogen bonding with surface hydroxyl groups of silica particles. An increase in melt viscosity due to the addition of silica 380 should also contribute to the thermal stability of solid dispersion. Viscosity effect is probably attributable to inhibition of the diffusion process involved in nuclei growth for crystallization.

Figure 5 shows the IR spectra of pure IMC crystal, pure IMC glass and a IMC–silica 380 (1 : 2) solid dispersion. Some of the peaks of the solid dispersion were different from those of the plain crystal, but the spectrum of the solid dispersion was similar to that observed for amorphous glass in that it had less sharp peaks. Three weak bands superimposed on the broad band at 2500—2800 cm^{-1}, due to the OH stretching vibration, disappeared or were markedly weakened, and two strong bands due to the CO stretching vibration at 1680—1720 cm^{-1} showed a frequency lowering of 3—4 cm^{-1}. The strong bands at 440—490, 1000—1250 and 3450 cm^{-1} of the solid dispersion are attributed to silica 380. The hydrogen bondings between carboxylic acid groups in IMC molecules form a dimer in the crystal packing.[6)] The change of IR spectra thus suggests that the state of hydrogen bonding in a solid dispersion is different from that in crystal. The results for GRIS samples are shown in Fig. 6. The IR spectra of amorphous GRIS

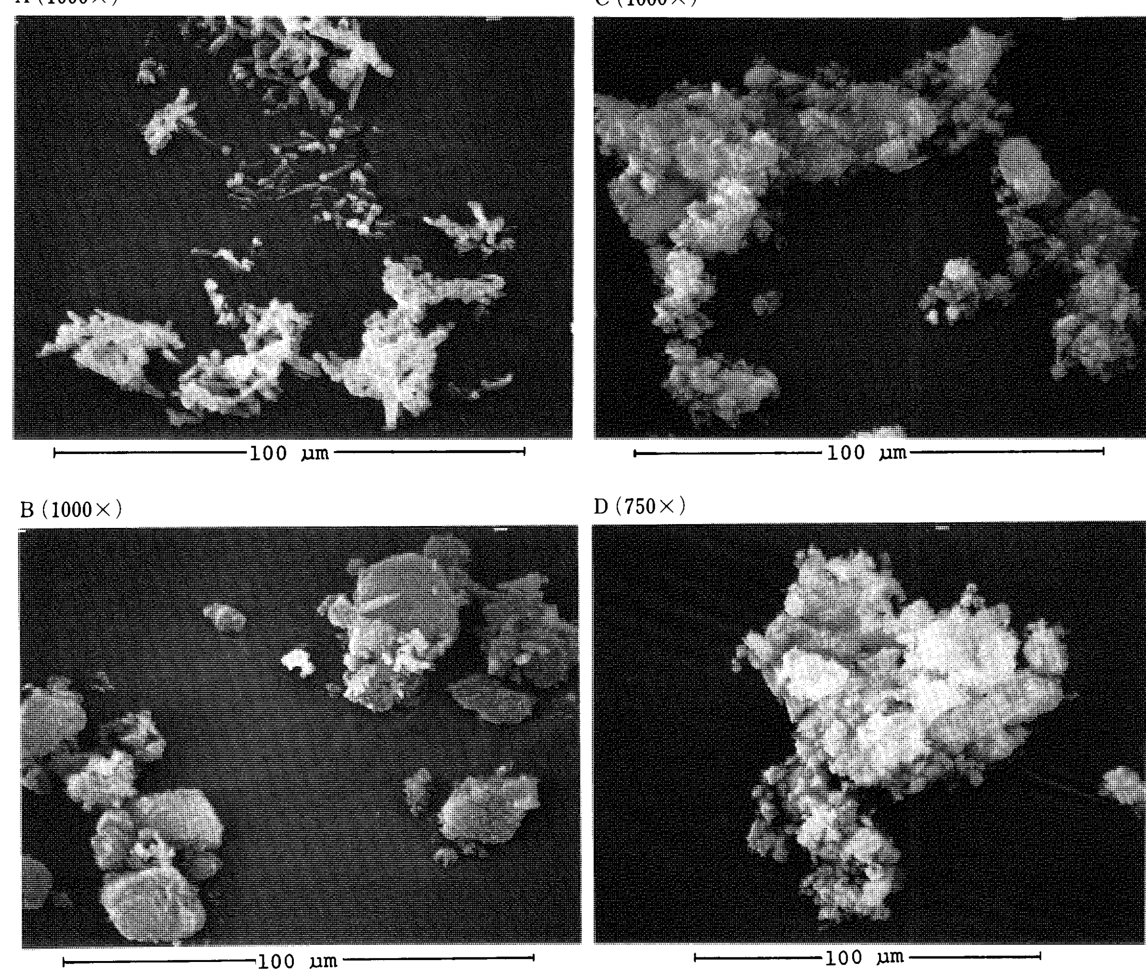

Fig. 8. Scanning Electron Micrographs of GRIS in Various Forms

A, crystalline GRIS; B, GRIS–silica 380 (1 : 2) physical mixture; C, GRIS–silica 380 (1 : 2) triturated mixture; D, GRIS–silica 380 (1 : 2) solid dispersion.

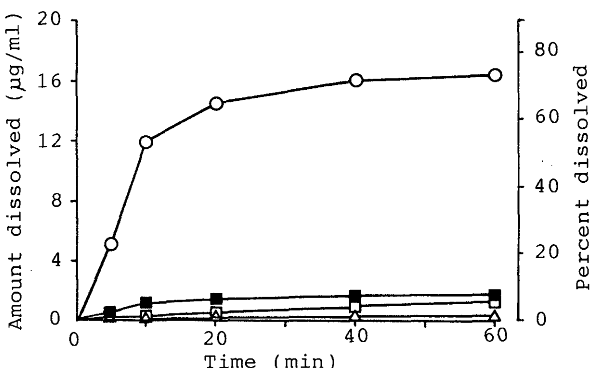

Fig. 9. Dissolution Profiles of IMC in Various Forms in JP XI 1st Fluid (pH 1.2) at 37 °C

△, IMC form I; □, IMC–silica 380 (1 : 2) physical mixture; ■, IMC–silica 380 (1 : 2) triturated mixture; ○, IMC–silica 380 (1 : 2) solid dispersion.

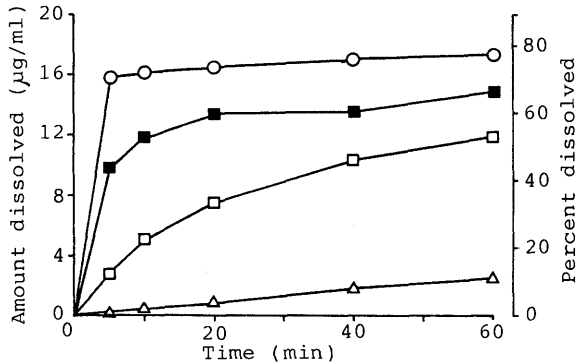

Fig. 10. Dissolution Profiles of GRIS in Various Forms in JP XI 1st Fluid (pH 1.2) at 37 °C

△, crystalline GRIS; □, GRIS–silica 380 (1 : 2) physical mixture; ■, GRIS–silica 380 (1 : 2) triturated mixture; ○, GRIS–silica 380 (1 : 2) solid dispersion.

and GRIS–silica 380 (1 : 2) solid dispersion were similar, but showed less sharp peaks than the pure crystalline GRIS.

Scanning electron microscopy (Fig. 7) of the IMC samples revealed the following: the physical mixture consisted of IMC crystal and aggregated silica particles, indicating that the intact drug crystal was randomly distributed in the inert carrier; the triturated mixture had IMC crystals to which the silica particles adhered homogeneously; and the solid dispersion showed none of the characteristics observed in the IMC crystal, but was in a state of aggregated microfine spherical particles. Similar results were obtained for the GRIS–silica 380 system (Fig. 8).

Figure 9 shows the dissolution behavior of IMC from various samples. A dramatic increase in the dissolution rate was observed for the solid dispersion relative to the pure crystalline IMC, and to the physical and triturated mixtures.

Figure 10 shows the dissolution profiles of GRIS preparations. The dissolution rate increased in the order physical mixture, triturated mixture, and solid dispersion.

In the case of IMC, the physical and triturated mixtures showed only slight improvement in the dissolution rate, while the physical and triturated mixtures of GRIS exhibited considerable improvement.

Several factors probably control the dissolution process of solid dispersion: surface area, solubility, transformation rate from amorphous to crystalline state, change of surface area caused by crystallization, and wettability. Further study is required to clarify the actual mechanism of rapid dissolution of the solid dispersion.

Fumed silicon dioxide has been reported to increase the dissolution rate of poorly water-soluble drugs when it is used as a carrier for either surface adsorption[7a–b] or solid dispersion.[7c] The surface adsorption was carried out by solvent disposition of drugs on porous or non-porous silicone dioxide with a large specific surface area. This method has the advantage, from the formulation point of view, of having a high drug–silicon dioxide ratio (10 : 1),[7a] but the disadvantage of using organic solvents. In particular, stable IMC form I is converted to metastable form II during the preparation by this method.[7b] A solid dispersion was obtained by simple mixing or by fusion of a drug with porous silicon dioxide, as reported by Kim *et al.*,[7c] who reported, however, that the drug showed no interaction with non-porous silicon dioxide.

The present study shows that non-porous silicon dioxide can be used to prepare solid dispersions of poorly water-soluble, glass-forming drugs. Porous silicon dioxide should also be an interesting carrier and may provide further information on the drug–silica solid dispersions. The advantage of a drug–silica solid dispersion is that it not only accomplishes fast release of the drug, but also can be used in formulations of solid dosage forms without grinding because it is a powder form. The merits of the melting method used for preparing the solid dispersion are its simplicity and economy; its demerit is that it is not applicable for drugs that decompose or evaporate during the fusion process, as discussed by Chiou and Riegelman.[8] Further study is necessary to clarify the effect of aging under various storage conditions on the fast-release characteristics and on the transformation from the amorphous to the crystalline state, since the amorphous state is a thermodynamically unstable form.

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A New Coumarin Glucoside from *Daphne arisanensis*

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A new coumarin glucoside named daphneside was isolated along with two known coumarin glucosides, daphnin and daphnetin-8-glucoside, and two known phenylpropanoid glucosides, syringin and syringinoside, from a water-soluble fraction of *Daphne arisanensis* HAYATA (Thymelaeaceae) collected in Taiwan. The structure of daphneside was elucidated by spectroscopic and chemical methods.

Keywords Thymelaeaceae; *Daphne arisanensis*; coumarin; phenylpropanoid; glucoside; daphneside; HPLC; CD

Our continuous study on the constituents of the Thymelaeaceae plants[2)] resulted in the isolation of a new coumarin glucoside named daphneside along with four known compounds (daphnin, daphnetin-8-glucoside, syringin, and syringinoside) from a water-soluble fraction of *Daphne arisanensis* HAYATA collected in Taiwan. This paper deals with the isolation and structure elucidation of daphneside by means of spectroscopic and chemical methods.

Results and Discussion

The methanol extract of the bark and root of the plant was subjected successively to partition to afford the fractions soluble in *n*-hexane, benzene, ethyl acetate, *n*-butanol and water. The water-soluble fraction was charged on an Amberlite XAD-2 resin column. Furthermore, the fraction eluted with 70% aqueous ethanol was separated by a combination of reversed-phase medium pressure and high-performance liquid chromatographies (MPLC and HPLC) to give five cmpounds (**1**—**5**).

Compound **1**, named daphneside, was obtained as colorless needles, mp 237 °C (dec.), $[\alpha]_D$ −17.1°. The molecular formula was determined as $C_{21}H_{26}O_{14}$ from the high-resolution fast atom bombardment mass spectrum (HR-FAB-MS). In the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra, the signals at δ_H 5.30 (1H, d, J=7.6 Hz), 5.34 (1H, d, J=7.6 Hz), and δ_C 102.6 (d), 105.4 (d) suggested the presence of two sugar moieties in the molecule. Furthermore, the signals at δ_H 6.47 (1H, d, J=9.5 Hz), 7.34 (1H, d, J=8.8 Hz), 7.44 (1H, d, J=8.8 Hz), 8.02 (1H, d, J=9.5 Hz), and δ_C 115.3 (d), 117.7 (d), 127.3 (d), 147.9 (d), 165.6 (s), coupled with the infrared (IR) absorptions at ν 1736, 1610 cm^{-1} suggested the presence of a coumarin skeleton. In fact the hydrolysis of daphneside (**1**) with 5% hydrochloric acid afforded daphnetin (**6**).[3)] This result suggested that the structure of daphneside may be daphnetin-7,8-di-*O*-saccharide (**1**), daphnetin-7-*O*-disaccharide (**7**) or daphnetin-8-*O*-disaccharide (**8**).

Recently, Nakanishi *et al.*[4)] reported the microscale structure determination of oligosaccharides using circular dichroism (CD) measurements for the identification of the component monosaccharides, linkage pattern, and absolute configuration.

Daphneside was esterified with *p*-bromobenzoyl chloride in the presence of silver trifluoromethanesulfonate (AgOTf) and 4-dimethylaminopyridine (DMAP) in pyridine to give daphneside octakis (*p*-bromobenzoate) (**9**) whose structure was confirmed by FAB-MS (m/z 1966.4 $(M+H)^+$). The benzoate (**9**) was subjected to a series of operations [i) HBr cleavage in $BrCH_2COOH$, ii) glycosidation with silver acetate and methanol, iii) deprotection with thiourea, iv) *p*-methoxycinnamoylation]. The structure **1** gives only a per-*p*-bromobenzoylated monosaccharide as a degradation product. On the other hand, the structure **2** or **3** gives a *p*-methoxycinnamoylated monosaccharide *p*-bromobenzoate as well as a per-*p*-bromobenzoated monosaccharide. The HPLC of the degradation product showed only one peak,

R₁O / OR₂ / OH / MeO / OMe / OR₃

1: $R_1=R_2=\beta$-D-glucopyranosyl
3: $R_1=\beta$-D-glucopyranosyl, R_2=H
5: R_1=H, $R_2=\beta$-D-glucopyranosyl
6: $R_1=R_2$=H
7: R_1=sugar-sugar, R_2=H
8: R_1=H, R_2=sugar-sugar

2: R_3=6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl
4: $R_3=\beta$-D-glucopyranosyl

Chart 1

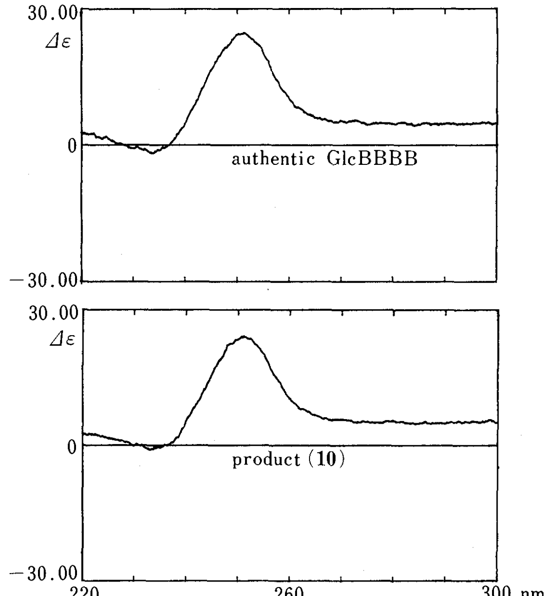

Fig. 1. CD Spectra of Methyl β-D-Glucopyranoside Tetrakis(*p*-bromobenzoate) (GlcBBBB) (**10**) in Acetonitrile

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which was identified as methyl β-glucopyranoside tetrakis (p-bromobenzoate) (**10**) by comparison of the retention time (t_R 5.95) and FAB-MS (m/z 927 $(M+H)^+$), and no other peak corresponding to p-methoxycinnamoylated monosaccharide. Furthermore, the CD spectrum of this product after HPLC purification was in excellent agreement with one of the synthetic standard from D-glucose as shown in Fig. 1.[4] As discussed above, the structure of daphneside should be represented as daphnetin-7,8-di-O-β-D-glucopyranoside (**1**).

The mass and ^{1}H-NMR spectra of compounds **3** and **5** were very similar to each other and both compounds gave the same products, daphnetin (**6**) and D-glucose, on hydrolysis with 5% hydrochloric acid. These results suggested **3** and **5** to be daphnetin monoglucosides. The linkage position of glucose was determined by a nuclear Overhauser effect (NOE) experiment. On irradiation of the anomeric proton (δ_H 4.92) in **3**, NOE (6.6%) was observed at H-6 (δ_H 7.03). Therefore, compound **3** and **5** were characterized as daphnetin-7-O-β-D-glucopyranoside (daphnin) and daphnetin-8-O-β-D-glucopyranoside, respectively.[5,6]

Compounds **2** and **4** have a common structure, the dimethoxyphenyl propenol moiety. Compound **2** has two sugar moieties (δ_H 4.24 (1H, d, $J=7.8$ Hz) and 4.87 (1H, d, $J=7.6$ Hz)) and compound **4** has one sugar moiety (δ_H 4.87 (1H, d, $J=7.6$ Hz)). These data are consistent with those of syringinoside (**2**) and syringin (**4**), respectively.[2]

Recently Chinese chemists reported that daphnin (**3**) inhibited rabbit platelet agrregation, prolonged the clotting time of blood, and reduced rat platelet adhesion.[7] Daphnetin (**6**) is slightly soluble in water, and daphnetin monoglucosides (**3** and **5**) are poorly soluble in water, but daphneside (**1**) is freely soluble in water. So we examined **1** for biological activity.

Experimental

General Procedures IR spectra were recorded on a JASCO FT-IR 5000 infrared spectrophotometer in KBr disk, ultraviolet (UV) spectra on a JASCO UVIDEC-610, optical rotations on a JASCO DIP-181 polarimeter at 25 °C, CD spectra on a JASCO J-600 spectropolarimeter in MeCN solution with $c=1\times10^{-5}$ M, and ^{1}H- and ^{13}C-NMR spectra on a JEOL GX-400 NMR spectrometer. MS (electron impact (EI) and FAB) spectra were measured on a Hitachi M-80 or JEOL HX-110 mass spectrometer. HPLC was carried out with a JASCO BIP-I (detector: JASCO UVIDEC-100-V, 254 nm).

Extraction The bark and root of the plant (1.0 kg) collected in the south of Taiwan in 1986 were immersed in MeOH (5 l) at room temperature for 2 weeks. Evaporation of MeOH under reduced pressure afforded a brownish residue (152 g), which was partitioned 3 times between hexane (each 400 ml) and 50% aqueous MeOH (1500 ml). The aqueous MeOH solution was concentrated to half the initial volume under reduced pressure, followed by dilution with water (500 ml). The aqueous solution was extracted 3 times successively with benzene (each 400 ml), AcOEt (each 400 ml), and BuOH (each 300 ml). The yields of the hexane-, benzene-, AcOEt-, BuOH-, and water-soluble fractions were 8.4, 1.0, 14.8, 15.9, 107.2 g, respectively.

Isolation A part of the above water-soluble fraction (60 g) was chromatographed on Amberlite XAD-2 (500 ml) with H_2O and then 70% aqueous EtOH to give fractions (53.3, 4.1 g). The second fraction (4.1 g) eluted with aqueous EtOH was repeatedly chromatographed on reversed-phase silica gel (Develosil C8-30/50, i.d. 25×250 mm), using MeOH–H_2O (30:70 and 15:85) to give the crude cmpounds. Each crude cmpound was purified by HPLC (Develosil C8-5, i.d. 10×250 mm), MeOH–H_2O (20:80), to give pure **1** (18 mg), **2** (15 mg), **3** (18 mg), **4** (66 mg), and **5** (12 mg).

Compound **1**: Colorless needles, mp 237 °C (dec.). $[\alpha]_D$ −17.1° ($c=0.29$, H_2O). HR FAB-MS m/z: Found 525.1205, $(C_{21}H_{26}O_{14}+Na)^+$ requires 525.1221. UV λ_{max} (MeOH) nm (ε): 304 (11200), 253 (3700), 205 (33800). IR ν (KBr): 3300 br., 1736, 1610 cm^{-1}. ^{1}H-NMR (D_2O): 3.45—4.05 (12H, complex), 5.30 (1H, d, $J=7.6$ Hz), 5.34 (1H, d, $J=7.6$ Hz), 6.47 (1H, d, $J=9.5$ Hz), 7.34 (1H, d, $J=8.8$ Hz), 7.44 (1H, d, $J=8.8$ Hz), 8.02 (1H, d, $J=9.5$ Hz). ^{13}C-NMR (D_2O): 62.8 (t), 62.8 (t), 71.57 (d), 71.61 (d), 75.1 (d), 76.0 (d), 77.8 (d), 77.9 (d), 78.6 (d), 78.6 (d), 102.6 (d), 105.4 (d), 115.3 (d), 115.7 (s), 117.7 (d), 127.3 (d), 134.4 (s), 147.9 (d), 149.5 (s), 154.2 (s), 165.6 (s).

Compound **3**: $[\alpha]_D$ −92.4° ($c=0.13$, CH_3OH). FAB-MS m/z: 341 $(M+H)^+$. ^{1}H-NMR (CD_3OD): 3.35—3.60 (4H, complex), 3.71 (1H, dd, $J=12.0$, 5.1 Hz), 3.90 (1H, dd, $J=12.0$, 2.0 Hz), 4.92 (1H, d, $J=7.6$ Hz), 6.28 (1H, d, $J=9.5$ Hz), 7.03 (1H, d, $J=8.8$ Hz), 7.21 (1H, d, $J=8.8$ Hz), 7.86 (1H, d, $J=9.5$ Hz). This was identical with an authentic sample of daphnetin-7-glucopyranoside.[5]

Compound **5**: $[\alpha]_D$ +31.0° ($c=0.14$, CH_3OH). FAB-MS m/z: 341 $(M+H)^+$. ^{1}H-NMR (CD_3OD): 3.40—3.60 (4H, complex), 3.72 (1H, dd, $J=12.0$, 4.6 Hz), 3.80 (1H, dd, $J=12.0$, 2.4 Hz), 4.87 (1H, d, $J=8.1$ Hz), 6.22 (1H, d, $J=9.5$ Hz), 6.55 (1H, d, $J=8.6$ Hz), 7.29 (1H, d, $J=8.6$ Hz), 7.86 (1H, d, $J=9.5$ Hz). This was identical with an authentic sample of daphnetin-8-glucopyranoside.[6]

Compound **2**: $[\alpha]_D$ −38.1° ($c=0.17$, H_2O). FAB-MS m/z: 535 $(M+H)^+$. ^{1}H-NMR (CD_3OD): 3.86 (6H, s), 4.22 (2H, dd, $J=5.6$, 1.5 Hz), 4.24 (1H, d, $J=7.8$ Hz), 4.87 (1H, d, $J=7.6$ Hz), 6.33 (1H, dt, $J=15.9$, 5.6 Hz), 6.55 (1H, dt, $J=15.9$, 1.5 Hz), 6.75 (2H, s). This was identical with an authentic sample of syringinoside.[2]

Compound **4**: $[\alpha]_D$ −16.1° ($c=0.11$, H_2O). FAB-MS m/z: 373 $(M+H)^+$. ^{1}H-NMR (CD_3OD): 3.86 (6H, s), 4.22 (2H, dd, $J=5.6$, 1.7 Hz), 4.87 (1H, d, $J=7.6$ Hz), 6.32 (1H, dt, $J=15.9$, 5.6 Hz), 6.54 (1H, dt, $J=15.9$, 1.7 Hz), 6.75 (2H, s). This was identical with an authentic sample of syringin.[2]

Bromobenzoylation of Daphneside (1) A mixture of daphneside (**1**) (2.2 mg, 4.38 μmol), p-bromobenzoyl chloride (9.2 mg, 42.0 μmol), AgOTf (10.8 mg, 42.0 μmol), and DMAP (1 crystal) in pyridine (500 μl) was stirred overnight at room temperature. After dilution with $CHCl_3$ the reaction mixture was passed through a SiO_2 pipet column and eluted with $CHCl_3$. The eluate was concentrated and the residue was separated by preparative thin layer chromatography (TLC) (Merck 5744, $CHCl_3$–benzene; 2:1) to give the desired compound (**9**) (6.1 mg). FAB-MS m/z: 1966.4 $(C_{77}H_{51}O_{22}Br_8+H)^+$. The molecular ion distribution pattern was in complete agreement with the theoretical pattern.

Preparation of Methyl β-Glucopyranoside Tetrakis(p-bromobenzoate) (10) A mixture of methyl β-D-glucopyranoside (Nacalai Tesque, 15.9 mg, 81.9 μmol), p-bromobenzoyl chloride (86.2 mg, 393 μmol), AgOTf (101 mg, 393 μmol), and DMAP (2 mg) in pyridine (0.1 ml) was stirred overnight at room temperature. After dilution with $CHCl_3$, the reaction mixture was separated by SiO_2 column chromatography and preparative TLC (Merck 13895, benzene) to give the desired compound (**10**) (41 mg). FAB-MS m/z: 927 $(C_{37}H_{26}O_{10}Br_4+H)^+$. The molecular ion distribution pattern was in excellent agreement with the theoretical pattern. ^{1}H-NMR ($CDCl_3$): 3.54 (3H, s), 4.14 (1H, complex), 4.50 (1H, dd, $J=4.9$, 12.2 Hz), 4.64 (1H, dd, $J=3.4$, 12.2 Hz), 4.75 (1H, d, $J=8.1$ Hz), 5.47 (1H, dd, $J=8.1$, 9.8 Hz), 5.63 (1H, dd, $J=9.8$, 9.8 Hz), 5.82 (1H, dd, $J=9.5$, 9.78 Hz), 7.43 (2H, d, $J=8.5$ Hz), 7.48 (2H, d, $J=8.5$ Hz), 7.52 (2H, d, $J=8.5$ Hz), 7.54 (2H, d, $J=8.5$ Hz), 7.67 (2H, d, $J=8.5$ Hz), 7.72 (2H, d, $J=8.5$ Hz), 7.80 (2H, d, $J=8.5$ Hz), 7.85 (2H, d, $J=8.5$ Hz).

Cleavage Reaction of Daphneside Octakis(p-bromobenzoate) (9) and Glycosidation Reaction of the Products Under an argon atmosphere, bromoacetyl bromide (250 μl, 2.87 mmol) and water (50 μl, 2.77 mmol) were added to daphneside octakis (p-bromobenzoate) (**9**) (2.2 mg, 1.1 μmol) in a special glass tube[4] at −78 °C. The vessel was sealed with a Teflon cap, and the mixture was stirred at room temperature for 12 h, then cooled again to −78 °C and the Teflon valve was carefully opened (HBr gas is released when the seal is broken). HBr gas was removed *in vacuo* under aspirator pressure for 5 min and then under high vacuum for 30 min. The resulting solid was treated with AgOAc (10 mg) in MeOH (0.2 ml) at room temperature for 1 h in the dark under an argon atmosphere. Silver salts were removed by filtration and the filtrate was concentrated. The resulting solid residue was suspended in hexane–EtOAc (2:1, 1 ml) and passed through a Pasteur pipett filled with a slurry of neutral Al_2O_3 in hexane–EtOAc (2:1). The Al_2O_3 column was washed with EtOAc (5 ml), and the eluate and washings were combined and concentrated to give a residue, which was lyophilized with benzene (0.2 ml).

Deprotection and Cinnamoylation Reactions Thiourea (3 mg) was added to a solution of the product mixture in CH_2Cl_2–MeOH (2:1, 0.3 ml), and

the mixture was stirred at room temperature for 2 h. $AgNO_3$ (10 mg) in CH_3CN (0.5 ml) was then added with stirring for an additional 5 min. The mixture was diluted with CH_2Cl_2 (3 ml) and passed through a Pasteur pipet filled with SiO_2 (0.5 g). The SiO_2 column was washed with CH_2Cl_2–MeOH (9 : 1, 10 ml), and the eluate and washings were concentrated to dryness, then lyophilized with benzene (0.2 ml) to give an amorphous powder.

p-Methoxycinnamoyl chloride (5 mg), AgOTf (5 mg) and DMAP (1 crystal) were added to a solution of the product in pyridine (0.2 ml) under an argon atmosphere. The reaction was allowed to proceed at room temperature for 12 h in the dark, then water (1 drop) was added, and the mixture was stirred for an additional 1 h. The reaction mixture was concentrated to dryness, suspended in hexane–EtOAc (2 : 1, 1 ml), then passed through a Pasteur pipet filled with 1 g of a neutral Al_2O_3 slurry in hexane–EtOAc (2 : 1). The Al_2O_3 column was washed with EtOAc (5 ml), and the eluate and washings were concentrated to afford a residue, which was separated by HPLC (column, Cosmosil 5SL, i.d. 4.6 × 150 mm; solvent, hexane–EtOAc (80 : 20); flow rate, 1.0 ml/min). t_R 5.95 min. FAB-MS *m/z*: 927 $(M+H)^+$. The molecular ion distribution pattern was identical with that of **10**. UV λ_{max} (CH_3CN): 243 nm. This product was concluded to be methyl *β*-glucopyranoside tetrakis(*p*-bromobenzoate) (**10**).

Acknowledgement The authors wish to thank Emeritus Professor Y. Hirata (Nagoya University) for his encouragement, Professors F. Murai and M. Tagawa (Aichi Medical University) for supplying an authentic sample of daphnetin-8-glucoside, and Sunbor Grant for financial support.

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A Highly Stereoselective Synthesis of (3*S*,4*S*)-Statine and (3*S*,4*S*)-Cyclohexylstatine[1)]

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The title compounds, which are synthetic intermediates of renin inhibitors, could be prepared from (*S*)-leucine and (*S*)-phenylalanine, respectively, by employing highly stereoselective aldol reactions of *O*-methyl-*O*-trimethylsilyl ketene acetal with an (*S*)-α-amino aldehyde in the presence of titanium(IV) chloride as a key step. Maximum diastereoselectivity of the aldol reaction was found to be more than 95:5.

Keywords renin inhibitor; (3*S*,4*S*)-statine; (3*S*,4*S*)-cyclohexylstatine; aldol reaction; ketene acetal; (*S*)-α-amide aldehyde; Lewis acid; titanium(IV) chloride; chelation-control

Renin is a highly specific enzyme operating in the cascade leading to the release of the hypertensive substance, angiotensin II, and plays a key role in the regulation of blood pressure, as does the angiotensin-converting enzyme (ACE).[2)] Thus, renin cleaves the Leu–Val peptide bond in angiotensinogen, producing a decapeptide, angiotensinogen I, which can be converted to angiotensin II by ACE. With the aim of developing a novel class of antihypertensive agents, intensive studies are currently being conducted on renin inhibitors having lower molecular weights.[2d,3)]

Since the natural peptide, pepstatin, was found to contain (3*S*,4*S*)-statine (**1a**) as the key component related to its inhibitory activity against renin,[4)] numerous synthetic peptide mimics bearing **1a** have been explored as potential renin inhibitors.[5)] Recently, some peptide-like compounds which contain (3*S*,4*S*)-cyclohexylstatine (**1b**) in place of **1a** were also found to exhibit promising profiles as candidate antihypertensive agents.[6)]

Numerous synthetic routes to **1** have been reported.[7,8)] While the aldol reaction of an enolate of an acetic acid derivative with an (*S*)-α-amido aldehyde such as **2**, accessible from (*S*)-leucine (**3a**) or (*S*)-phenylalanine (**3b**), is anticipated to be one of the simplest synthetic routes to **1**, it is well recognized that the addition reaction employing an achiral enolate results in no significant stereoselection[7a,9)] and the desired stereoselectivity can be realized only by using a less readily accessible chiral enolate.[7a,10)]

Fig. 1

We have now found that the aldol reaction of *O*-methyl-*O*-trimethylsilyl ketene acetal (**4**)[11)] with **2** proceeds in a highly stereoselective manner in the presence of titanium(IV) chloride ($TiCl_4$), affording a mixture of the addition products (**5** and **6**) in which the desired adduct (**5**) is predominant (**5**:**6**≥95:5). Removal of the protective groups involved in **5** under acidic conditions readily produced **1**. This report details an efficient synthesis of **1** explored by employing the highly stereoselective aldol reaction of **4** with **2**.[12)]

Thus, as shown in Chart 1, (*S*)-**2a** could be readily prepared from (*S*)-**3a** in 4 steps according to the procedure reported for the synthesis of (*S*)-**2b** from (*S*)-**3b**.[13)] Inexpensive isopropoxycarbonyl group was used as a convenient protective group for the amino function.[13)] After some preliminary experiments, it was found that the reaction of (*S*)-**2a** with **4** could proceed highly stereoselectively in the presence of $TiCl_4$ at −78 °C in dichloromethane, giving a mixture of **5a** and **6a** in 89% combined yield (Table I, run 1). The formation ratio of **5a** to **6a** was estimated as 95:5 by analyzing the mixture of **5a** and **6a** by gas liquid

a: $R^1 = R^2 = Me_2CH$
b: R^1 = cyclo-C_6H_{11}, $R^2 = C_6H_5$

(a) MeOH, $SOCl_2$ (a 100%, b 96%); (b) iso-PrOCOCl, K_2CO_3, CH_2Cl_2 (a 87%) or iso-PrOCOCl, Et_3N, THF (b 91%); (c) $NaBH_4$, LiCl, THF-EtOH (a 100%, b 98%) (d) H_2 (4 atm), Rh-Al_2O_3, AcOH-MeOH (100%); (e) SO_3·Py, DMSO, Et_3N, toluene (a 79%, b 78%); (f) CH_2=C(OMe)OTMS (4); see, the text and Table I; (g) 2,2-dimethoxypropane, *p*-TsOH, CH_2Cl_2 (7a 86%, 7b 88%, 8a 85%, 8b 91%); (h) 6 M HCl, AcOEt, 100°C; Dowex AG 50W (H^+ form) (1a 88%, 1b 93%)

Chart 1

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TABLE I. The Aldol Reaction of (S)-α-Amido Aldehyde (**2**) with O-Methyl-O-trimethylsilyl Ketene Acetal (**4**) under Various Conditions

Run	Compound	R^1	Reaction conditions			Yield of **5** and **6** (%)	Ratio of **5** to **6**[b]
			Lewis acid (eq)	Solv.[a]	Temp. (°C)		
1	**2a**	Me_2CH	$TiCl_4$ (1.5)	CH_2Cl_2	−78	89	95: 5
2	**2a**	Me_2CH	$TiCl_4$ (1.5)	CH_2Cl_2	−25	82	82:18
3	**2a**	Me_2CH	$TiCl_4$ (1.5)	PhMe	−78	80	80:20
4	**2a**	Me_2CH	$BF_3 \cdot Et_2O$ (1.2)	CH_2Cl_2	−78	72	80:20
5	**2a**	Me_2CH	ZnI_2 (1.2)	THF	−40→0[c]	45 (61)[d]	90:10
6	**2a**	Me_2CH	Eu(fod)$_3$ (0.05)	CH_2Cl_2	0→r.t.[e]	48 (29)[d]	93: 7
7	**2b**	cyclo-C_6H_{11}	$TiCl_4$ (1.5)	CH_2Cl_2	−78	95	96: 4

a) Dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), or toluene (PhMe). *b*) Determined by GLC analysis (5% Silar 10C, 190 °C) of a mixture of **5** and **6**. *c*) The reaction temperature was gradually raised from −40 °C to 0 °C over 1.0 h. *d*) Recovery of the starting material (**2a**). *e*) The reaction mixture was stirred at 0 °C for 4 h, then at room temperature for 12 h. r.t. = room temperature.

chromatography (GLC).

Treatments of separated **5a** and **6a** with 2,2-dimethoxypropane in the presence of p-toluenesulfonic acid produced the 2,2-dimethyl-1,3-oxazoline derivatives (**7a** and **8a**) in 86% and 85% yields, respectively. The relative stereochemistries of **5a** and **6a** could be rigorously determined by measuring the proton nuclear magnetic resonance (^{1}H-NMR) spectra of **7a** and **8a**. Since the vicinal protons (Ha and Hb) involved in the 1,3-oxazolidine moieties of **7a** and **8a**, were found to exhibit the coupling constants of 0.0 and 5.0 Hz, repectively, the *trans* and *cis* stereochemistries could be assigned to **7a** and **8a**. Thus, **5a** and **6a** have the desired (3S,4S)- and the undesired (3R,4S)-configurations, respectively.[14)]

The aldol reaction of **2a** with **4** was further examined under various reaction conditions. Some representative results are shown in Table I. Thus, when the reaction was carried out at higher temperature, such as −25 °C, or in toluene, the mixture of **5a** and **6a** was produced in lower yields and stereoselectivities (runs 2 and 3). The use of boron trifluoride–etherate ($BF_3 \cdot Et_2O$) in place of $TiCl_4$ also decreased the yield and stereoselectivity (run 4). Similarly to the aldol reaction using $TiCl_4$, the reactions employing zinc(II) iodide (ZnI_2) or tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato)europium(III) [Eu(fod)$_3$] as a Lewis acid were found to take place in a highly stereoselective manner even at higher reaction temperature (runs 5 and 6). In these cases, however, the recovery of the starting material (**2a**) was always observed, probably due to the lower reaction rate.

When **2b** prepared from (S)-**3b**[13)] was treated with **4** under the best reaction conditions established with **2a**, a mixture of **5b** and **6b** could be obtained in 95% combined yield (Table I, run 7). The ratio of **5b** to **6b** was similarly determined as 96:4 by GLC analysis. The relative stereochemistries of **5b** and **6b** were assigned as (3S,4S)- and (3R,3S)-configurations, respectively, by measuring the ^{1}H-NMR spectra of **7b** and **8b** derived from **5b** and **6b** in the same manner as described for **5a** and **6a**.

The preferential formation of **5** may be rationalized in terms of the chelation-controlled mechanism depicted in Fig. 2. This is interesting in view of the reported result that an α-alkoxy aldehyde is not suceptible to similar chelation-controlled diastereofacial selection.[15)]

The major aldol product (**5a**) isolated by column chromatography was subjected to acidic hydrolysis, giving **1a**,

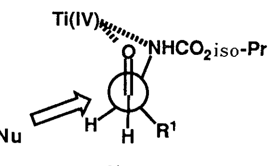

Fig. 2

mp 202—205 °C (dec.) and $[\alpha]_D^{20}$ −20.4° (water), in 88% yield.[7f,16)] Treatment of the other major product (**5b**) under the same conditions as for **5a** gave **1b**, mp 213—216 °C (dec.) and $[\alpha]_D^{20}$ −25.3° (1 M hydrochloric acid), in 93% yield.[7c,h)]

Thus, we have succeeded in developing an efficient synthetic route to **1a** and **1b** by employing the aldol reaction of (S)-**2** with **4** in the presence of $TiCl_4$. Taking into account its directness and operational simplicity, the overall process seems to be one of the most practical synthetic methods available for **1a, b**.

Experimental

All melting points were determined with a Yamato MP-21 melting point apparatus and are uncorrected. Measuremens of optical rotations were carried out using a Horiba SEPA-200 automatic digital polarimeter. Infrared (IR) spectra measurements were performed with a JASCO A-200 IR spectrometer. ^{1}H-NMR spectra were measured with a Hitachi R-90H spectrometer (90 MHz), a Bruker AM 400 spectrometer (400 MHz), and a JEOL JNM-GX 500 spectrometer (500 MHz). All signals are expressed as ppm downfield from tetramethylsilane, used as an internal standard (δ value). The following abbreviations are used: singlet (s), doublet (d), triplet (t), multiplet (m), broad (br). Mass spectra (MS) were taken with a Hitachi RMU-6MG mass spectrometer and a JEOL JMS-D 300 mass spectrometer. Unless otherwise noted, all reactions were performed in anhydrous solvents. Wako gel C-200 was used as an adsorbent for column chromatography.

(S)-N-(Isopropoxycarbonyl)leucinol According to the reported procedure,[17)] methyl (S)-leucinate could be prepared in 100% yield by treating **3a** with thionyl chloride in MeOH. Potassium carbonate (228 mg, 1.65 mmol) and isopropyl chloroformate (0.23 ml, 1.98 mmol) were added to a solution of methyl (S)-leucinate (239 mg, 1.65 mmol) in CH_2Cl_2 (5 ml) at 0 °C and the mixture was stirred for 1 h at the same temperature. After quenching of the reaction with saturated $NaHCO_3$ solution, the resulting mixture was extracted with ether. The ethereal extracts were combined, washed with brine, dried over anhydrous $MgSO_4$, then concentrated *in vacuo*. The residue was purified by column chromatography (hexane : AcOEt = 4 : 1) to give methyl (S)-N-(isopropoxycarbonyl)leucinate (333 mg, 87%). $[\alpha]_D^{20}$ −4.9° (c = 1.53, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.95 (6H, m), 1.23 (6H, two d, J = each 6.1 Hz), 1.3—1.8 (3H, m), 3.73 (3H, s), 4.40 (1H, m), 4.90 (2H, m and doubled q, J = each 6.2 Hz). IR ($CHCl_3$): 3460, 2970, 1730, 1710, 1510, 1110 cm^{-1}. MS m/z: 231 (M^+), 172, 86, 43.

A suspension of methyl (S)-N-(isopropoxycarbonyl)leucinate (333 mg, 1.44 mmol), lithium chloride (183 mg, 4.32 mmol), and sodium borohydride (163 mg, 4.32 mmol) in a mixture of tetrahydrofuran (THF) (4 ml) and

EtOH (6 ml) was stirred for 5 h at room temperature. The mixture was concentrated *in vacuo* and the residue was diluted with 1 M HCl under ice cooling. The acidic mixture was extracted with AcOEt. The combined AcOEt extracts were washed with brine, dried over anhydrous $MgSO_4$, then concentrated *in vacuo*. The residue was chromatographed (hexane : AcOEt = 2 : 1) to give (*S*)-*N*-(isopropoxycarbonyl)leucinol (292 mg, 100%). $[\alpha]_D^{20}$ −30.9° (*c* = 1.05, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (6H, two d, *J* = each 6.4 Hz), 1.23 (6H, two d, *J* = each 6.2 Hz), 1.3—2.0 (3H, m), 2.45 (1H, br s), 3.65 (3H, m), 4.70 (1H, m), 4.90 (1H, doubled q, *J* = each 6.2 Hz). IR ($CHCl_3$): 3460, 2970, 1700, 1510, 1110 cm^{-1}. MS *m/z*: 204 (M^+ + 1), 172, 130, 86, 43. *Anal.* Calcd for $C_{10}H_{21}NO_3$: C, 59.08; H, 10.41; N, 6.89. Found: C, 58.93; H, 10.40; N, 6.91.

(*S*)-*N*-(Isopropoxycarbonyl)phenylalaninol According to the reported procedure,[17] methyl (*S*)-phenylalabinate hydrochloride could be prepared in 96% yield by treating **3b** with thionyl chloride in MeOH. Acylation of methyl (*S*)-phenylalaninate hydrochloride (3.90 g, 13 mmol) with isopropyl chloroformate (2.3 ml, 20 mmol) and triethylamine (5.5 ml, 39 mmol) under the same conditions as reported[13b] gave methyl (*S*)-*N*-(isopropoxycarbonyl)phenylalaninate (4.35 g, 91%) as a colorless solid. $[\alpha]_D^{20}$ +55.4° (*c* = 1.32, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.21 (6H, two d, *J* = each 6 Hz), 3.11 (2H, d, *J* = 6 Hz), 3.72 (3H, s), 4.5—5.2 (2H, m), 4.92 (1H, doubled q, *J* = each 6 Hz), 7.04—7.48 (5H, m). IR (KBr): 1740, 1685 cm^{-1}. MS *m/z*: 266 (M^+ + 1), 206, 162, 131, 120.

Treatment of methyl (*S*)-*N*-(isopropoxycarbonyl)phenylalaninate (2.52 g, 9.5 mmol) with lithium chloride (1.22 g, 29 mmol) and sodium borohydride (1.09 g, 29 mmol) in a mixture of THF (16 ml) and EtOH (25 ml) in a similar manner to that reported[13b] gave (*S*)-*N*-(isopropoxycarbonyl)phenylalaninol (2.21 g, 98%) as a colorless solid after purification by column chromatography (hexane : AcOEt = 5 : 1→3 : 1). $[\alpha]_D^{20}$ −25.2° (*c* = 1.03, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.21 (6H, two d, *J* = each 6 Hz), 1.96—2.30 (1H, m), 2.88 (2H, d, *J* = 7 Hz), 3.44—4.16 (3H, m), 4.65—5.13 (1H, m), 4.92 (1H, doubled q, *J* = each 6 Hz), 7.05—7.47 (5H, m). IR (KBr): 1690 cm^{-1}. MS *m/z*: 237 (M^+), 206, 146, 120. *Anal.* Calcd for $C_{13}H_{19}NO_3$: C, 65.80; H, 8.07; N, 5.90. Found: C, 65.99; H, 8.03; N, 5.81.

(*S*)-*N*-(Isopropoxycarbonyl)leucinal (2a) Sulfur trioxide pyridine complex (1.32 g, 8.30 mmol) was added slowly to a stirred solution of (*S*)-*N*-(isopropoxycarbonyl)leucinol (281 mg, 1.38 mmol) and triethylamine (1.16 ml, 8.30 mmol) in a mixture of toluene (1 ml) and dimethylsulfoxide (DMSO) (1.8 ml) under cooling (<10 °C) and the mixture was stirred for 30 min at room temperature. After quenching of the reaction with ice and water, the mixture was extracted with AcOEt and the combined ethyl acetate extracts were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (hexane : AcOEt = 8 : 1) to afford **2a** as a colorless oil (219 mg, 79%). $[\alpha]_D^{20}$ +34.4° (*c* = 0.964, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.97 (6H, m), 1.24 (6H, two d, *J* = each 6.2 Hz), 1.3—1.9 (3H, m), 4.25 (1H, m), 4.92 (2H, m and doubled q, *J* = each 6.2 Hz) 9.59 (1H, s). IR ($CHCl_3$): 3460, 2980, 2720, 1720, 1710, 1500, 1380, 1110 cm^{-1}. MS *m/z*: 172, 130, 86, 43.

(*S*)-3-Cyclohexyl-2-(isopropoxycarbonyl)aminopropanal (2b) According to the reported method,[13)] a mixture of (*S*)-*N*-(isopropoxycarbonyl)phenylalaninol (2.05 g, 8.6 mmol), Rh-Al_2O_3 (403 mg), and AcOH (0.6 ml) in MeOH (6 ml) was stirred under a hydrogen atmosphere (4 atm) for 5 h at room temperature. The mixture was filtered to remove the catalyst and the combined filtrates were concentrated *in vacuo*. The residue was purified by column chromatography (hexane: AcOEt = 5 : 1) to give (*S*)-3-cyclohexyl-2-(isopropoxycarbonyl)aminopropanol as an oil (2.09 g, 100 %). $[\alpha]_D^{20}$ −26.4° (*c* = 1.78, $CHCl_3$) [lit.,[13b] $[\alpha]_D^{23}$ −27.2° (*c* = 1.06, $CHCl_3$)]. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (6H, two d, *J* = each 6 Hz), 0.71—2.13 (13H, m), 3.36—4.02 (3H, m), 4.51—5.15 (1H, m), 4.93 (1H, doubled q, *J* = each 6 Hz). IR (neat): 1690 cm^{-1}. MS *m/z*: 244 (M^+ + 1), 212, 170, 126. *Anal.* Calcd for $C_{13}H_{25}NO_3 \cdot 0.1H_2O$: C, 63.69; H, 10.36; N, 5.71. Found: C, 63.56; H, 10.39; N, 5.75.

Sulfur trioxide pyridine complex (491 mg, 3.1 mmol) were added slowly to a stirred solution of (*S*)-3-cyclohexyl-2-(isopropoxycarbonyl)aminopropanol (138 mg, 0.57 mmol) and triethylamine (0.43 ml, 3.1 mmol) in a mixture of toluene (0.33 ml) and DMSO (0.67 ml) under cooling (<10 °C) in a similar manner to that reported.[13b] The resulting mixture was stirred for 20 min at room temperature. After quenching of the reaction with ice and water, the mixture was extracted with AcOEt. The combined organic extracts were washed successively with water and brine, dried over anhydrous $MgSO_4$, then concentrated *in vacuo*. The residue was purified by column chromatography (hexane : AcOEt = 10 : 1→5 : 1) to afford **3b** as a colorless oil (107 mg, 78%). $[\alpha]_D^{20}$ +26.6° (*c* = 0.939, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.60—2.06 (13H, m), 1.23 (6H, two d, *J* = each 7 Hz), 4.03—4.43 (1H, m), 4.93 (1H, doubled q, *J* = each 6.2 Hz), 5.20—5.52 (1H, m), 9.56 (1H, s). IR (neat): 1730, 1690 cm^{-1}. MS *m/z*: 242 (M^+ + 1), 212, 170, 126.

Methyl (3*S*,4*S*)-3-Hydroxy-6-methyl-4-(isopropoxycarbonyl)aminoheptanoate (5a) and Its (3*R*,4*S*)-Isomer (6a) Experimental procedures for Table I, runs 1 and 4, will be described as representative examples.

a) Table I, Run 1: A solution of $TiCl_4$ in CH_2Cl_2 (1.0 M solution, 0.065 mmol) was added slowly to a suspension of **2a** (8.7 mg, 0.043 mmol), **4** (19 mg, 0.13 mmol), and molecular sieves 4Å (4 mg) in CH_2Cl_2 (0.5 ml) at −78 °C under an argon atmosphere. After being stirred for 1 h at the same temperature, the mixture was quenched with a small amount of saturated $NaHCO_3$ solution and filtered through a pad of Celite. The combined filtrate and washing were concentrated *in vacuo*. The residue obtained as an oil was purified by column chromatography (hexane : AcOEt = 4 : 1), giving a mixture of **5** and **6** (10.6 mg, 89%). The ratio of **5a** to **6a** was determined as 95 : 5 by GLC analysis of the residue (5% Silar 10C, 190 °C). For the physical and spectral data of **5a** and **6a**, see b).

b) Table I, Run 4: A solution of $BF_3 \cdot Et_2O$ in CH_2Cl_2 (1.0 M solution, 0.17 mmol) was added slowly to a suspension of **2a** (28.9 mg, 0.144 mmol), **4** (63 mg, 0.43 mmol), and molecular sieves 4Å (5 mg) in CH_2Cl_2 (0.5 ml) at −78 °C under an argon atmosphere. After being stirred for 1 h at the same temperature, the mixture was quenched with saturated $NaHCO_3$ solution and extracted with AcOEt. The combined organic extracts were washed with 1 M HCl and brine, dried over anhydrous $MgSO_4$, then concentrated *in vacuo*. The residue, obtained as an oil, was purified by column chromatography (hexane : AcOEt = 4 : 1) to yield **5a** (19.4 mg), **6a** (6.5 mg), and a mixture of **5a** and **6a** (2.6 mg) (the totol yield of **5a** and **6a**, 72%). The ratio of **5a** to **6a** was calculated as 80 : 20 by GLC analysis of the residue (5% Silar 10C, 190 °C). **5a**: oil, $[\alpha]_D^{20}$ −43.2° (*c* = 1.07, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (6H, m), 1.23 (6H, two d, *J* = each 6.2 Hz), 1.35 (1H, m), 1.54 (1H, m), 1.66 (1H, m), 2.53 (2H, m), 3.30 (1H, br s), 3.67 (1H, m), 3.72 (3H, s), 4.03 (1H, br s), 4.82 (1H, br d, *J* = 9.7 Hz), 4.98 (1H, doubled q, *J* = each 6.2 Hz). IR ($CHCl_3$): 3470, 2980, 1710, 1505, 1110 cm^{-1}. MS *m/z*: 276 (M^+ + 1), 216, 172, 130, 86, 43. *Anal.* Calcd for C, 56.70; H, 9.15; N, 5.09. Found: C, 56.50; H, 9.33; N, 5.18. **6a**: mp 63—65 °C (from Et_2O–hexane), $[\alpha]_D^{20}$ −29.8° (*c* = 0.650, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (6H, two d, *J* = each 6.5 Hz), 1.23 (6H, two d, *J* = each 6.1 Hz), 1.34 (2H, m), 1.68 (1H, m), 2.50 (2H, m), 3.35 (1H, br s), 3.71 (4H, s and m), 4.02 (1H, br s), 4.62 (1H, br d, *J* = 7.8 Hz), 4.89 (1H, doubled q, *J* = each 6.1 Hz). IR ($CHCl_3$): 3460, 2970, 1710, 1505, 1110 cm^{-1}. MS *m/z*: 316 (M^+ + 1), 256, 212, 170, 126, 71, 43. *Anal.* Calcd for $C_{13}H_{25}NO_5 \cdot 0.1H_2O$: C, 56.34; H, 9.17; N, 5.05. Found: C, 56.21; H, 9.12; N, 4.93.

Methyl (3*S*,4*S*)-5-Cyclohexyl-3-hydroxy-4-(isopropoxycarbonyl)aminopentanoate (5b) and Its (3*R*,4*S*)-Isomer (6b) Table I, Run 7: A solution of $TiCl_4$ in CH_2Cl_2 (1.0 M solution, 0.15 mmol) was added slowly to a suspension of **2b** (23.7 mg, 0.098 mmol), **4** (43 mg, 0.30 mmol), and molecular sieves 4Å (10 mg) in CH_2Cl_2 (1 ml) at −78 °C under an argon atmosphere. The mixture was stirred for 1 h at the same temperature, and, after quenching of the reaction with a small amount of saturated $NaHCO_3$ solution, filtered through a pad of Celite. The combined filtrate and washings were concentrated *in vacuo*. The residue, obtained as an oil, was purified by column chromatography (hexane : AcOEt = 4 : 1), affording **5b** (27.4 mg) and **6b** (2 mg) (the total yield of **5b** and **6b**, 95%). The ratio of **5b** to **6b** was determined as 96 : 4 by GLC analysis of the residue (5% Silar 10C, 190 °C). **5b**: oil, $[\alpha]_D^{20}$ −36.6° (*c* = 1.11, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.8—1.1 (2H, m), 1.1—1.3 (2H, m), 1.23 (6H, two d, *J* = each 6.2 Hz), 1.3—1.6 (2H, m), 1.66 (4H, m), 1.82 (1H, br s), 2.52 (2H, m) 3.24 (1H, br s), 3.69 (1H, m), 3.71 (3H, s), 4.02 (1H, br d, *J* = 6.7 Hz), 4.77 (1H, br d, *J* = 9.8 Hz), 4.88 (1H, doubled q, *J* = each 6.2 Hz). IR ($CHCl_3$): 3470, 2940, 2870, 1710, 1505, 1440, 1110 cm^{-1}. MS *m/z*: 316 (M^+ + 1), 256, 212, 170, 126, 100, 43. *Anal.* Calcd for $C_{16}H_{29}NO_5$: C, 60.93; H, 9.27; N, 4.44. Found: C, 60.60; H, 9.18; N, 4.34. **6b**: mp 73—74 °C (from hexane) and $[\alpha]_D^{20}$ −30.8° (*c* = 0.510, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.7—1.1 (2H, m), 1.23 (6H, two d, *J* = each 6.0 Hz), 1.1—1.5 (5H, m), 1.53—1.76 (4H, m), 1.83 (1H, br d, *J* = 13.0 Hz), 2.48 (2H, m), 3.38 (1H, br s), 3.71 (3H, s), 3.77 (1H, m), 4.02 (1H, m), 4.60 (1H, br d, *J* = 7.7 Hz), 4.89 (1H, doubled q, *J* = each 6.0 Hz). IR ($CHCl_3$): 3690, 3460, 2930, 2860, 1710, 1500, 1430, 1110 cm^{-1}. MS *m/z*: 276, 216, 172, 130, 86, 43. *Anal.* Calcd for $C_{16}H_{29}NO_5 \cdot 0.2H_2O$: C, 60.24; H, 9.29; N, 4.39. Found: C, 60.33; H, 9.31; N, 4.25.

Methyl 1-[(4*S*,5*S*)-4-(2-Methyl)propyl-2,2-dimethyl-3-isopropoxycarbonyl-1,3-oxazolidin-5-yl]acetate (7a) A mixture of **5a** (16.4 mg, 0.060 mmol),

2,2-dimethoxypropane (12.4 mg, 0.12 mmol), and *p*-toluenesulfonic acid (1.1 mg) in CH_2Cl_2 (2 ml) was stirred for 6 h at room temperature. The reaction was quenched with saturated $NaHCO_3$ solution and the mixture was extracted with ether. The combined extracts were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purifed by column chromatography (hexane : AcOEt = 4 : 1), giving **7a** as an oil (16.2 mg, 86%). $[\alpha]_D^{20}$ +6.5° (c=0.430, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (6H, two d, J=each 6.3 Hz), 1.26, 1.27 (6H, two d, J=each 6.2 Hz), 1.5—1.7 (3H, m), 1.52, 1.61 (each 3H, s), 2.60 (1H, dd, J=6.5, 15.3 Hz), 2.68 (1H, dd, J=7.5, 15.3 Hz), 3.71 (3H, s), 3.62 (1H, m), 4.33 (1H, dd, J=6.5, 7.5 Hz), 4.94 (1H, m). IR ($CHCl_3$): 2970, 1730, 1690, 1400, 1110 1090 cm^{-1}. MS *m/z*: 300, 258, 214, 172, 140, 99, 43.

Methyl [(4*S*,5*R*)-4-(2-Methyl)propyl-2,2-dimethyl-3-isopropoxycarbonyl-1,3-oxazolidin-5-yl]acetate (8a) This compound was prepared as an oil in 85% yield (13.7 mg) from **6a** (14.1 mg, 0.051 mmol) by the same procedure as described for the preparation of **7a**. $[\alpha]_D^{20}$ −11.7° (c=0.426, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (6H, m), 1.25, 1.26 (6H, two d, J=each 6.1 Hz), 1.2—1.6 (3H, m), 1.56 (6H, s), 2.58 (1H, dd, J=6.8, 16.3 Hz), 2.68 (1H, br dd, J=7.3, 16.3 Hz), 3.72 (3H, s), 4.06 (1H, m), 4.44 (1H, ddd, J=5.0, 6.8, 7.3 Hz), 4.95 (1H, m). IR ($CHCl_3$): 2980, 1735, 1685, 1405, 1110, 1095 cm^{-1}. MS *m/z*: 300, 258, 214, 172, 43.

Methyl [(4*S*,5*S*)-4-Cyclohexylmethyl-2,2-dimethyl-3-isopropoxycarbonyl-1,3-oxazolidin-5-yl]acetate (7b) The same treatments of **5b** (16.8 mg, 0.0533 mmol) as described for the preparation of **7a** gave **7b** as an oil (16.7 mg, 88%) after purification by column chromatography. $[\alpha]_D^{20}$ + 3.8° (c=0.630, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.8—1.8 (13H, m), 1.21 (6H, two d, J=each 6.3 Hz), 1.46, 1.56 (6H, two s), 2.55 (1H, dd, J=6.5, 14 Hz), 2.63 (1H, dd, J=7.5, 14 Hz), 3.66 (3H, s), 3.78 (1H, m), 4.28 (1H, ddd, J=1.4, 6.5, 7.5 Hz), 4.88 (1H, br s). IR ($CHCl_3$): 3000, 2940, 2860, 1730, 1690, 1405, 1110 cm^{-1}. MS *m/z*: 340, 254, 215, 172, 70, 55, 43.

Methyl [(4*S*,5*R*)-4-Cyclohexylmethyl-2,2-dimethyl-3-isopropoxycarbonyl-1,3-oxazolidin-5-yl]acetate (8b) Treatments of **6b** (5.3 mg, 0.017 mmol) by the same procedure as described for the preparation of **8a** gave **8b** (5.4 mg, 91%) as an oil after purification by column chromatography. $[\alpha]_D^{20}$ −9.6° (c=0.499, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.8—1.9 (13H, m), 1.25, 1.27 (6H, two d, J=each 6.2 Hz), 1.55 (6H, s), 2.58 (1H, dd, J=6.8, 16.4 Hz), 2.68 (1H, dd, J=7.5, 16.4 Hz), 3.72 (3H, s), 4.07 (1H, m), 4.44 (1H, ddd, J=4.8, 6.8, 7.5 Hz), 4.92 (1H, m). IR ($CHCl_3$): 3010, 2950, 2880, 1735, 1685, 1410, 1110 cm^{-1}. MS *m/z*: 340, 254, 180, 172, 55, 43.

(3*S*,4*S*)-Statine (1a) A mixture of **5a** (40.7 mg, 0.15 mmol), 6 M HCl (2 ml), and AcOEt (0.5 ml) was heated at 100 °C for 13 h. After concentration *in vacuo*, the residue was purified by column chromatography using an ion exchange resin [Dowex AG50W-X2, 1 M pyridine–AcOH buffer solution (pH 5)], giving **1a** as colorless crystals (22.9 mg, 88%). A pure sample of **1a** was obtained by recrystallization from water–EtOH, mp 202—205 °C (dec.), $[\alpha]_D^{20}$ −20.4° (c=0.501, H_2O) [ref. 7*f* mp 214—215 °C (dec.), $[\alpha]_D^{20}$ −20.8° (c=2.3, H_2O); ref. 16 mp 201—203 °C (dec.), $[\alpha]_D$ −20° (c=0.64, H_2O)]. ^{1}H-NMR (D_2O) δ: 0.94 (6H, two d, J= each 5.7 Hz), 1.2—1.9 (3H, m), 2.48 (1H, dd, 7.0, 15 Hz), 2.53 (1H, dd, J=5.1, 15 Hz), 3.30 (1H, m), 4.10 (1H, m). IR (KBr): 3440, 3220, 2970, 2890, 1600, 1550, 1510, 1430, 1390, 1170, 720 cm^{-1}. MS *m/z*: 176 (M^+ + 1), 172, 157, 140, 118, 100, 86, 40.

(3*S*,4*S*)-Cyclohexylstatine (1b) A mixture of **5b** (16.0 mg, 0.051 mmol), 6 M HCl (2 ml), and AcOEt (0.5 ml) was heated at 100 °C for 8 h. The reaction mixture was treated in the same manner as described for the preparation of **1a** to give **1b** (10.2 mg, 93%) as colorless crystals after concentration of the eluate from a column of ion exchange resin. A pure sample of **1b** was precipitated from a solution of **2a** in 1M HCl by neutralizing with 1 M NaOH. mp 213—216 °C (dec.), $[\alpha]_D^{20}$ −25.3° (c=0.435, 1 M HCl). [ref. 7*c* mp 230—231 °C (dec.), $[\alpha]_D^{25}$ −26.2° (c= 1.0, 1 M HCl); ref. 7*h* mp 214—216 °C (dec.), $[\alpha]_D^{23}$ −22.49° (c=0.979, 1 M HCl)]. ^{1}H-NMR (D_2O) δ: 0.8—2.0 (13H, m), 2.55 (2H, m), 3.35 (1H, m), 4.00 (1H, m). IR (KBr): 3430, 3220, 2950, 2880, 1610, 1550, 1510, 1440, 1385, 1335, 1115, 1070, 1035, 980, 885 cm^{-1}. MS *m/z*: 216 (M^+ + 1), 197, 126, 100, 55.

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Synthesis and Anti-peptic Activity of Compounds Related to the Metabolites of Sodium 3-Ethyl-7-isopropyl-1-azulenesulfonate (KT1-32)

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The metabolites of sodium 3-ethyl-7-isopropyl-1-azulenesulfonate (KT1-32, 1), a candidate as an anti-ulcer drug, and related compounds were synthesized. The effects of the compounds on anti-peptic activity were determined as compared to that of 1.

Keywords metabolite; sodium 3-ethyl-7-isopropyl-1-azulenesulfonate (KT1-32); anti-peptic activity

In previous papers, we described the synthesis and the anti-ulcer activity of new sodium alkylazulenesulfonate derivatives.[1,2] Among them, sodium 3-ethyl-7-isopropyl-1-azulenesulfonate (KT1-32, **1**) was found to possess potent inhibitory action against Shay-ulcer and anti-peptic activity. Furthermore, **1** was extremely stable upon heating. Thus, compound **1** was selected for evaluation in humans as a promising novel agent for the therapy of peptic ulcer. It is now under clinical trial. Metabolic studies are very important in the development of new drugs and are essential to assess the safety and efficacy of medicines. Recently, a study on the metabolism of **1** in biological fluids of rat was presented and three mono-hydroxylated derivatives (**3**, **4** and **5**) and two di-hidroxylated derivatives (**6** and **7**) and a hydrogen sulfated derivative (**8**) were proposed as the metabolites.[3] In this paper, we wish to report the synthesis of compounds (**2**—**7**) related to the metabolites of **1** and their anti-peptic activities.

Results and Discussion

Synthesis of Hydroxyazulene Derivatives Oxidation[5] of **1** with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) in aqueous acetone afforded 3-acetylazulene **9**. Reduction of **9** with sodium borohydride ($NaBH_4$) afforded **2**. Attempts to afford **5** by several oxidizing agents failed. But when a solution of **1** in water was irradiated was 30 W low-pressure Hg lamp,[6] compound **5** was obtained in a 10% yield. Compound **7** was prepared from **3** in the same manner. Deprotection of **10**[1] with aluminium chloride ($AlCl_3$) in anisole afforded **3**. Bromination[4] of **11**[1] with *N*-bromosuccinimide (NBS) in dichloromethane afforded allylic bromide **13** in a 24% yield. Treatment of **13** with sodium acetate in *N*,*N*-dimethylformamide (DMF) afforded allylic acetate **15**. Catalytic hydrogenation of **15** with 5% palladium–carbon (Pd–C) afforded acetate **17**. Decarboxylation of **17** with anhydrous phosphoric acid afforded **19**. Protection

TABLE I. Anti-peptic Activity of the Metabolites **3**—**7** and the Reference Compounds **2** and **1**

Compd. No.	Concentration (mM)	Inhibition(%)	IC_{50}[a] (mM)
3	1	16.0	3.8
	3	37.2	
	10	100.0	
4	1	1.1	2.9
	3	51.1	
	10	81.9	
5	0.3	13.8	1.5
	1	30.9	
	3	73.4	
6	10	41.5	>10.0
7	10	33.8	>10.0
2	1	7.5	2.6
	3	66.7	
	10	93.6	
1	0.1	12.3	0.5
	0.3	21.7	
	1	80.7	

a) IC_{50} values were calculated from the concentration–inhibition relations by the method of least squares.

1 (KT 1 - 32)

8

	R_1	R_2	R_3	R_4
2	H	OH	H	H
3	OH	H	H	H
4	H	H	H	OH
5	H	H	OH	H
6	OH	H	H	OH
7	OH	H	OH	H

Chart 1

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Chart 2

of **19** with acetic anhydride in pyridine afforded acetate **21** in a 93.5% yield. Sulfonation of **21** with a pyridine–sulfur trioxide complex in benzene followed by treatment with a sodium methoxide afforded sodium salt **4**. Compound **6** was prepared from **12** in the same manner as in the case of **4**.

Biological Activities Table I summarizes the anti-peptic activity of the metabolites **3**—**7** and the reference compounds **2** and **1**. All the synthesized derivatives showed lower anti-peptic activity than **1**. Dihydroxylated derivatives (**6** and **7**) exhibited greatly decreased activity. The decrease of anti-peptic activity by the introduction of a hydroxy group at the alkyl side chain on azulene suggests that the alkyl groups without hydroxy groups may play important roles in the manifestation of anti-peptic activity.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 270-30. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured at 90 MHz on a Hitachi R-90H Fourier transform NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given as values (ppm): s, singlet; d, doublet; dd, double doublet; ddd, doublet of doublets of doublets; t, triplet; q, quartet; br, broad; sept, septet; m, multiplet. Mass spectra (MS) were taken on a Hitachi M-80B spectrometer. For column chromatography, silica gel (Merck, Kieselgel 60, 70—230 mesh) was used.

Sodium 3-Acetyl-7-isopropyl-1-azulenesulfonate (9) 2,3-Dichloro-5,6-dicyano-*p*-bezoquinone (DDQ) (16.6 g, 7.3×10^{-2} mol) was added to a stirred solution of **1**[1] (10.0 g, 3.3×10^{-2} mol) in 10% aqueous acetone (100 ml) and the mixture was stirred at room temperature for 1 h. The solvent was evaporated and dioxane (100 ml) was added to the residual material. DDQ-H was filtered off, and the filtrate was evaporated. The

residue was purified by column chromatography ($CHCl_3$: MeOH = 3 : 1) to give **9** (7.5 g, 72%) as red violet crystals. mp 158—166 °C (dec.). IR (KBr) cm^{-1}: 2950, 1650, 1200. ^1H-NMR (90 MHz, CD_3OD) δ: 1.43 (6H, d, iso-Pr–CH_3), 2.66 (3H, s, $COCH_3$), 3.28 (1H, sept, iso-Pr–CH), 7.77 (1H, dd, C_5-H), 7.98 (1H, dd, C_6-H), 8.58 (1H, s, C_2-H), 9.42 (1H, d, C_8-H), 9.75 (1H, d, C_4-H).

Sodium 3-(1-Hydroxyethyl)-7-isopropyl-1-azulenesulfonate (2) $NaBH_4$ (1.0 g, 2.6×10^{-2} mol) was added to a stirred solution of **9** (6.0 g, 1.9×10^{-2} mol) in H_2O (50 ml) at room temperature. The reaction mixture was stirred at room temperature for 4 h, and extracted with *n*-BuOH. After removal of the solvent, the residue was passed through a column of TSK gel (Toyopearl HW-40) (H_2O) to give **2** (5.1 g, 85%)as violet crystals. mp > 280 °C. IR (KBr) cm^{-1}: 3400, 2950, 1420, 1180. ^1H-NMR (90 MHz, CD_3OD) δ: 1.39 (6H, d, iso-Pr–CH_3), 1.60 (3H, d, CH–C$\underline{H}_3$), 3.20 (1H, sept, iso-Pr–CH), 5.54 (1H, q, C$\underline{H}$–OH), 7.36 (1H, dd, C_5-H), 7.78 (1H, dd, C_6-H), 8.20 (1H, s, C_2-H), 8.50 (1H, d, C_4-H), 9.27 (1H, d, C_8-H).

Sodium 3-Ethyl-7-(1-hydroxy-1-methylethyl)-1-azulenesulfonate (5) A solution of **1**[1] (10 g, 3.3×10^{-2} mol) in H_2O (500 ml) was irradiated with a 30 W low-pressure Hg lamp for 68 h under O_2-gas bubbling at room temperature and then concentrated *in vacuo*. The residue was purified by Sephadex G-10 column chromatography (H_2O) to give **5** (1.1 g, 10%) as violet crystals. mp > 280 °C. IR (KBr) cm^{-1}: 3400, 1210. ^1H-NMR (90 MHz, CD_3OD) δ: 1.36 (3H, t, Et–CH_3), 1.72 (6H, s, C(OH)C$\underline{H}_3$), 3.04 (2H, q, Et–CH_2), 7.32 (1H, dd, C_5-H), 8.05 (1H, s, C_2-H), 8.18 (1H, ddd, C_6-H), 8.36 (1H, dd, C_4-H), 9.46 (1H, d, C_8-H).

Sodium 3-(2-Hydroxyethyl)-7-(1-hydroxy-1-methylethyl)-1-azulenesulfonate (7) Violet crystals. mp > 280 °C. IR (KBr) cm^{-1}: 3450, 1200. ^1H-NMR (90 MHz, CD_3OD) δ: 1.70 (6H, s, C(OH)C$\underline{H}_3$), 3.24 (2H, t, C$\underline{H}_2$–CH_2–OH), 3.83 (2H, t, CH_2–C$\underline{H}_2$–OH), 7.32 (1H, dd, C_5-H), 8.05 (1H, s, C_2-H), 8.18 (1H, ddd, C_6-H), 8.38 (1H, dd, C_4-H), 9.46 (1H, d, C_8-H).

Sodium 3-(2-Hydroxyethyl)-7-isopropyl-1-azulenesulfonate (3) A solution of **10** (10.6 g, 2.6×10^{-2} mol) in anisole (50 ml) was added to a suspension of $AlCl_3$ (10.4 g, 7.8×10^{-2} mol) in anisole (50 ml) at room temperature and the mixture was stirred for 2 h. The reaction mixture was poured into ice water (50 ml) and the mixture was extracted with *n*-BuOH. The extract was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the residue was purified by column chromatography ($CHCl_3$: MeOH = 3 : 1) to give **3** (7.8 g, 95%) as violet crystals. mp > 280 °C. IR (KBr) cm^{-1}: 3400, 2950, 1160. ^1H-NMR (90 MHz, CD_3OD) δ: 1.40 (6H, d, iso-Pr–CH_3), 3.20 (1H, sept, iso-Pr–CH), 3.24 (2H, t, C$\underline{H}_2$–CH_2–OH), 3.84 (2H, t, CH_2–C$\underline{H}_2$–OH), 7.30 (1H, dd, C_5-H), 7.73 (1H, ddd, C_6-H), 8.06 (1H, s, C_2-H), 8.36 (1H, dd, C_4-H), 9.23 (1H, d, C_8-H).

Methyl 7-(1-Bromomethylvinyl)-3-ethyl-1-azulenecarboxylate (13) *N*-Bromosuccinimide (NBS) (83 g, 4.6×10^{-1} mol) was added at 0 °C to a stirred solution of **11**[1] (60 g, 2.3×10^{-1} mol) in CH_2Cl_2 (300 ml) and the mixture was stirred at room temperature for 2 h. The solution was poured into water, and the organic layer was washed with brine, dried and evaporated. The residue was purified by column chromatography (hexane : EtOAc = 10 : 1) to give **13** (18.7 g, 24%) as a violet oil. MS *m/z*: 333 (M^+). IR (neat) cm^{-1}: 2950, 1700, 1460. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.35 (3H, t, Et–CH_3), 3.05 (2H, q, Et–CH_2), 3.90 (3H, s, $COOCH_3$), 4.50 (2H, s, Br–CH_2), 5.60 (2H, d, C = CH_2), 7.26 (1H, dd, C_5-H), 7.80 (1H, dd, C_6-H), 8.23 (1H, s, C_2-H), 8.30 (1H, d, C_4-H), 9.78 (1H, d, C_8-H).

Methyl 7-(1-Bromoethylvinyl)-3-(2-benzyloxyethyl)-1-azulenecarboxylate (14) A violet oil, 22.5% yield. MS *m/z*: 440(M^+). IR (neat) cm^{-1}: 2950, 1730, 1450. ^1H-NMR (90 MHz, $CDCl_3$) δ: 3.30 (2H, t, C$\underline{H}_2$–CH_2–OBzl), 3.80 (2H, t, CH_2–C$\underline{H}_2$–OBzl), 3.90 (3H, s, $COOCH_3$), 4.52 (2H, s, C$\underline{H}_2$–C_6H_5), 4.53 (2H, s, Br–CH_2), 5.60 (2H, d, C = CH_2), 7.28 (5H, s, C_6H_5), 7.40 (1H, dd, C_5-H), 7.85 (1H, dd, C_6-H), 8.26 (1H, s, C_2-H), 8.38 (1H, d, C_4-H), 9.78 (1H, d, C_8-H).

Methyl 7-(1-Acetoxymethylvinyl)-3-ethyl-1-azulenecarboxylate (15) A solution of sodium acetate (7.3 g, 8.9×10^{-2} mol) in DMF (20 ml) was gradually added to a stirred solution of **13** (15 g, 4.5×10^{-2} mol) in DMF (30 ml) at room temperature. After stirring for 10 h at 50 °C, the solution was poured into water and extracted with EtOAc. The extract was washed with brine, dried and evaporated. The residue was purified by column chromatography (hexane : EtOAc = 10 : 1) to give **15** (12.3 g, 87.0%) as a blue violet oil. MS *m/z*: 313 (M^+). IR (neat) cm^{-1}: 2950, 1740. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.38 (3H, t, Et–CH_3), 2.05, (3H, s, AcO), 3.02 (2H, q, Et–CH_2), 3.90 (3H, s, $COOCH_3$), 5.08 (2H, s, AcO–C$\underline{H}_2$), 5.50 (2H, d, C = CH_2), 7.35 (1H, dd, C_5-H), 7.78 (1H, dd, C_6-H), 8.23 (1H, s, C_2-H), 8.30 (1H, d, C_4-H), 9.78 (1H, d, C_8-H).

Methyl 7-(1-Acetoxymethylvinyl)-3-(2-benzyloxyethyl)-1-azulenecarboxylate (16) A violet oil, 80% yield. MS *m/z*: 418 (M^+), IR (neat) cm^{-1}: 2980, 1750, 1700. ^1H-NMR (90 MHz, $CDCl_3$) δ: 2.06 (3H, s, OAc), 3.30 (2H, t, C$\underline{H}_2$–CH_2–OBzl), 3.80 (2H, t, CH_2–C$\underline{H}_2$–OBzl), 3.90 (3H, s, $COOCH_3$), 4.52 (2H, s, C$\underline{H}_2$–C_6H_5), 5.10 (2H, s, C$\underline{H}_2$–OAc), 5.58 (2H, d, C = CH_2), 7.28 (5H, s, C_6H_5), 7.40 (1H, dd, C_5-H), 7.80 (1H, dd, C_6-H), 8.26 (1H, s, C_2-H), 8.36 (1H, d, C_4-H), 9.78 (1H, d, C_8-H).

Methyl 7-(2-Acetoxy-1-methylethyl)-3-ethyl-1-azulenecarboxylate (17) A solution of **15** (10 g, 3.2×10^{-2} mol) in EtOH (50 ml) was stirred with 5% Pd–C (200 mg) under H_2 gas at room temperature for 10 h. The catalyst was filtered off, and the solution was evaporated. The residue was purified by column chromatography (hexane : EtOAc = 10 : 1) to give **17** (7.8 g, 78%) as a violet oil. MS *m/z*: 314 (M^+). IR (neat) cm^{-1}: 2950, 1740. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.35 (3H, t, Et–CH_3), 1.42 (3H, d, CH–C$\underline{H}_3$), 1.95 (3H, s, OAc), 3.00 (2H, q, Et–CH_2), 3.35 (1H, m, C$\underline{H}$–CH_3), 3.90 (3H, s, $COOCH_3$), 4.30 (2H, d, CH–C$\underline{H}_2$–OAc), 7.30 (1H, dd, C_5-H), 7.62 (1H, dd, C_6-H), 8.20 (1H, s, C_2-H), 8.26 (1H, d, C_4-H), 9.63 (1H, d, C_8-H).

Methyl 7-(2-Acetoxy-1-methylethyl)-3-(2-benzyloxyethyl)-1-azulenecarboxylate (18) A violet oil, 75% yield. MS *m/z*: 420 (M^+) . IR (neat) cm^{-1}: 2950, 1740, 1700. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.47 (3H, d, CH–C$\underline{H}_3$), 1.98 (3H, s, OAc), 3.30 (2H, t, C$\underline{H}_2$–CH_2–OBzl), 3.32 (1H, m, C$\underline{H}$–CH_3), 3.80 (3H, t, CH_2–C$\underline{H}_2$–OBzl), 3.94 (3H, s, $COOCH_3$), 4.30 (2H, d, C$\underline{H}_2$–OAc), 4.54 (2H, s, C$\underline{H}_2$–C_6H_5), 7.30 (5H, s, C_6H_5), 7.40 (1H, dd, C_5-H) 7.69 (1H, dd, C_6-H), 8.25 (1H, s, C_2-H), 8.38 (1H, d, C_4-H), 9.68 (1H, d, C_8-H).

1-Ethyl-5-(2-hydroxy-1-methylethyl)azulene (19) The treatment of **17** with anhydrous phospholic acid in the same manner as described previously[1] afforded **19** as violet prisms. mp 62—63 °C. MS *m/z*: 214 (M^+). IR (KBr) cm^{-1}: 3250, 2950. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.34 (3H, d, CH–C$\underline{H}_3$), 1.35 (3H, t, Et–CH_3), 3.05 (2H, q, Et–CH_2), 3.20 (1H, m, C$\underline{H}$–CH_3), 3.78 (2H, d, C$\underline{H}_2$–OH), 7.05 (1H, dd, C_7-H), 7.25 (1H, d, C_3-H), 7.40 (1H, dd, C_6-H), 7.78 (1H, d, C_2-H), 8.16 (1H, d, C_4-H), 8.18 (1H, d, C_8-H).

1-(2-Hydroxyethyl)-5-(2-hydroxy-1-methylethyl)azulene (20) A violet oil, 78% yield. MS *m/z*: 230 (M^+). IR (neat) cm^{-1}: 3350, 2900, 1720. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.35 (3H, d, CH–C$\underline{H}_3$), 3.12 (1H, m, C$\underline{H}$–CH_3), 3.30 (2H, t, C$\underline{H}_2$–CH_2–OH), 3.78 (2H, d, CH–C$\underline{H}_2$–OH), 3.80 (2H, t, CH_2–C$\underline{H}_2$–OH), 7.00 (1H, dd, C_7-H), 7.26 (1H, d, C_3-H), 7.48 (1H, dd, C_6-H), 7.78 (1H, d, C_2-H), 8.20 (1H, d, C_4-H), 8.25 (1H, d, C_8-H).

5-(2-Acetoxy-1-methyl)-1-ethylazulene (21) Acetic anhydride (2.2 g, 2.2×10^{-2} mol) was added to a stirred ice-cooled solution of **19** (2.6 g, 1.1×10^{-2} mol) in dry pyridine (20 ml) and the mixture was stirred at 0 °C for 2 h. The mixture was poured into water, and extracted with EtOAc. The extract was washed with water, dried over anhydrous Na_2SO_4 and evaported. The residue was column chromatography (hexane) to give **21** (2.9 g, 93.5%) as a blue violet oil. MS *m/z*: 256 (M^+). IR (neat) cm^{-1}: 2950, 1730, 1450. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.37 (3H, t, Et–CH_3), 1.38 (6H, d, CH–C$\underline{H}_3$), 1.98 (3H, s, OAc), 3.04 (2H, q, Et–CH_2), 3.17 (1H, m, C$\underline{H}$–CH_3), 4.24 (2H, d, C$\underline{H}_2$–OAc), 7.06 (1H, dd, C_7-H), 7.26 (1H, d, C_3-H), 7.42 (1H, dd, C_6-H), 7.78 (1H, d, C_2-H), 8.16 (1H, d, C_4-H), 8.20 (1H, d, C_8-H).

1-(2-Acetoxyethyl)-5-(2-acetoxy-1-methylethyl)azulene (22) A blue oil, 83% yield. MS *m/z*: 314 (M^+). IR (neat) cm^{-1}: 2960, 1740, 1400. ^1H-NMR (90 MHz, $CDCl_3$) δ: 1.40 (3H, d, CH–C$\underline{H}_3$), 1.98 (3H, s, CH–CH_2–O$\underline{Ac}$), 2.03 (3H, s, CH_2–CH_2–O$\underline{Ac}$), 3.30 (1H, m, C$\underline{H}$–CH_3), 3.39 (2H, t, C$\underline{H}_2$–CH_2–OAc), 4.30 (2H, t, CH_2–C$\underline{H}_2$–OAc), 7.04 (1H, dd, C_7-H), 7.28 (2H, d, C_3-H), 7.48 (1H, dd, C_6-H), 7.80 (1H, d, C_3-H), 8.21 (1H, d, C_4-H), 8.26 (1H, d, C_8-H).

Sodium 3-Ethyl-7-(2-hydroxy-1-methylethyl)-1-azulenesulfonate (4) The treatment of **21** with pyridine–sulfur trioxide complex in the same manner as described previously[1] afforded **4** as violet crystals. mp > 280 °C. IR (KBr) cm^{-1}: 3400, 1200. ^1H-NMR (90 MHz, CD_3OD) δ: 1.37 (3H, t, Et–CH_3), 1.40 (3H, d, CH–CH_3), 3.04 (2H, q, Et–CH_2), 3.17 (1H, m, CH–CH_3), 3.74 (1H, dd, CH_2–OH), 3.86 (1H, dd, CH_2–OH), 7.28 (1H, dd, C_5-H), 7.70 (1H, dd, C_6-H), 8.06 (1H, s, C_2-H), 8.33 (1H, d, C_4-H), 9.15 (1H, d, C_8-H).

Sodium 3-(2-Hydroxyethyl)-7-(2-hydroxy-1-methylethyl)-1-azulenesulfonate (6) Violet crystals, mp > 280 °C. IR (KBr) cm^{-1}: 3400, 1650, 1300. ^1H-NMR (90 MHz, CD_3OD) δ: 1.40 (3H, d, CH–C$\underline{H}_3$), 3.16 (1H, m, C$\underline{H}$–CH_3), 3.23 (2H, t, C$\underline{H}_2$–CH_2–OH), 3.75 (2H, d, CH–C$\underline{H}_2$–OH), 3.82 (2H, t, CH_2–C$\underline{H}_2$–OH), 7.29 (1H, dd, C_5-H), 7.70 (1H, dd, C_6-H), 8.05 (1H, s, C_2-H), 8.37 (1H, d, C_4-H), 9.15 (1H, d, C_8-H).

Anti-peptic Activity Anti-peptic activity was measured according to the modified method of Thiemer *et al.*[7] A mixture of bovine serum albumin (BSA) 1 ml (5 mg/ml), 0.3 ml of 0.1 N HCl and 2 ml of water, with or without a test drug, was pre-incubated at 37 °C for 5 min. The reaction

was started by the addition of 0.5 ml of enzyme solution (10 μg pepsin/ml of 0.5 N HCl) and was stopped by the addition of 2 ml of 10% trichloroacetic acid after 10 min incubation at 37 °C. The amount of hydrolyzed BSA was measured according to the method of Udenfriend *et al.*[8] After centrifugation of the reaction mixture at 3000 rpm for 10 min, 0.1 ml aliquot of supernatant fraction was transferred to a test tube and 2 ml of 0.2 M boric acid (pH 9.2) was added followed by 1 ml of fluorescamine solution (0.3 mg fluorescamine/ml of acetone). Then the contents of the tube was mixed immediately and the fluorescence at 470 nm resulting from activation at 390 nm was measured in a spectrofluorometer. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = (A - B)/A \times 100$$

where A is the amount of hydrolysed BSA in the absence of a test drug and B is that in the presence of a test drug.

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Synthesis and Oral Activity of Pivaloyloxymethyl 7-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (ME1207) and Its Related Compound

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7-[2-(2-Aminothiazol-4-yl)-2(*Z*)-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylic acid (11, ME1206) and its 3-*trans* isomer (13) were prepared to test antibacterial activity. These compounds exhibited excellent antibacterial activity against both gram-positive and gram-negative bacteria, including β-lactamase producing strains.

The pivaloyloxymethyl esters (12 and 14) of the compounds (11 and 13) were prepared by esterification with pivaloyloxymethyl iodide. Among them, pivaloyloxymethyl 7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (12, ME1207) showed good urinary recovery after oral administration in mice.

Keywords cephalosporin; oral cephalosporin; *in vitro* antibacterial activity; structure–activity relationship; prodrug

Cephalosporins bearing 2-alkyloxyimino 2-(2-aminothiazol-4-yl) acetamido moieties as a C-7 side chain, which had broad and potent antibacterial activity against gram-positive and gram-negative bacteria, have been widely used for antibacterial chemotherapy. However, most of them are not suitable for oral administration because of their low absorption from the gastrointestinal tract, except for cefixime (CFIX)[1] and cefetram pivoxil (CFTM-PI).[2] Thus, the need still exists for development of a new orally active, semi-synthetic cephalosporin which exhibits potent and broad-spectrum antibacterial activity.

In a previous paper[3] relating to the antibacterial activity and oral absorption of 3-alkylthio-7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-(*O*-substituted oxyimino)acetamido]cephalosporins having various *O*-substituents of the oxime, we reported that the pivaloyloxymethyl ester of 7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3-methylthio-3-cephem-4-carboxylic acid had good *in vivo* efficacy against mice infection caused by *Escherichia coli* No. 29 and showed high urinary recovery after oral administration in mice. Although the free acid, active form of this cephalosporin showed excellent activity against gram-negative bacteria, it did not show satisfactory activity against gram-positive bacteria. In due course, we investigated a modification of the 3-substituent in the hope of improving the antibacterial activity against gram-positive bacteria while retaining high antibacterial activity against gram-negative bacteria. As a result, the introduction of heterocyclic substituted vinyl groups to C-3 was fruitful.[4] In particular, pivaloyloxymethyl 7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-4-(methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (**12**, ME1207)[5] showed excellent oral activity and ME1206 (**11**),[5] an active form of ME1207, showed potent and broad antibacterial activity against both gram-positive and gram-negative bacteria. This paper deals with the synthesis and structure–activity relationships of a new orally active cephalosporin, ME1207, and a 3-trans isomer (**14**) of ME1207.

Results and Discussion

Chemistry The *p*-methoxybenzyl 7-phenylacetamido-3-chloromethyl-3-cephem-4-carboxylate (**1**)[6] was converted to the corresponding triphenylphosphonium iodide (**2**) by treatment with NaI and PPh_3 in acetone in 90% yield. A Wittig reaction of **2** with 5-formyl-4-methylthiazole (**3**)[7] was carried out in a heterogeneous system of dichloromethane–water at room temperature in the presence of sodium bicarbonate to give an 84% yield of a mixture of the vinyl derivative **4** (*Z*, *cis* isomer) and **5** (*E*, *trans* isomer) in a ratio of 4.7 : 1. Each isomer could be separated by fractional recrystallization followed by column chromatography. The olefin geometry was determined on the basis of proton nuclear magnetic resonance (^{1}H-NMR) spectra; the major product having a smaller vinyl coupling constant (J = 11 Hz) was assigned to be the *Z* isomer, whereas the minor one with a larger coupling constant (J = 16 Hz) was the *E* isomer.

The phenylacetyl side chains of **4** and **5** were cleaved by a known imino-chloride method, followed by silicagel column chromatography to afford amino ester (**6**) and **7** in good yields, respectively. Compounds **6** and **7** were coupled with 2-(2-tritylaminothiazol-4-yl)-2(*Z*)-methoxyiminoacetic acid (**8**)[8] using $POCl_3$ as a coupling reagent to give the protected cephalosporins **9** (*Z* isomer) and **10** (*E* isomer), respectively.

Removal of the protective groups of **9** and **10** with CF_3COOH–anisole, and purification by Diaion HP-20 column chromatography gave new cephalosporins **11** and **13**, respectively. Alternately, the sodium salts (**11** and **13**) were treated with iodomethyl pivalate in dimethylformamide (DMF) to give the pivaloyloxymethyl esters (**12** and **14**) in good yields.

Biological Evaluation The minimum inhibitory concentrations (MICs) of the new cephalosporins (**11** and **13**) were determined by the twofold agar dilution method. The MICs values of these compounds against several gram-positive and gram-negative bacteria are summarized in Table I and compared with the values of CFIX, CFTM[2] and cefaclor (CCL).[9] These compound showed potent and broad antibacterial activity against both gram-positive and gram-negative bacteria. Especially, the activity of these compounds (**11** and **13**) against gram-positive bacteria was more potent than either CFIX, CFTM or CCL. The activity of compounds **11** and **13** against gram-negative bacteria was more potent than CCL and comparable to CFIX and CFTM. The effect of the stereochemistry of **11** (*Z* isomer) and **13** (*E* isomer) on the anti-

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PMB= CH_2–C_6H_4–OCH_3

POM= $CH_2OCOC(CH_3)_3$

Chart 1

TABLE I. *In Vitro* Activity of ME1206 (**11**) and Related Cephalosporins

Test organism	MIC (µg/ml)				
	ME1206 (**11**)	**13**	CFTM	CFIX	CCL
Staphylococcus aureus 606[a]	0.78	0.78	6.25	6.25	3.13
S. aureus 606 E-25	0.78	0.78	3.13	6.25	3.13
Bacillus subtilis ATCC 6633	0.20	0.39	0.78	50	0.20
Escherichia coli W3630 RGN823[a]	0.39	0.20	0.39	0.78	25
E. coli No. 29	0.39	0.39	0.39	0.20	1.56
Klebsiella pneumoniae GN69[a]	0.20	0.39	0.20	0.05	3.13
Salmonella typhi 0-901-W	0.05	0.10	0.05	<0.025	0.78
Proteus vulgaris GN76[b]	0.20	0.10	0.20	<0.025	>100
P. vulgaris GN76/C-1[b]	0.20	0.10	3.13	0.05	>100
Morganella morganii 1510/S-1	0.20	0.10	0.20	0.39	6.25
Shigella dysenteriae (shiga)	0.05	0.05	0.05	0.39	0.78
Enterobacter cloacae G-0008[b]	0.78	0.78	1.56	0.78	>100
Pseudomonas aeruginosa GN10362[b]	25	100	100	>100	>100

a) Penicillinase producing strain. *b*) Cephalosporinase producing strain.

bacterial activity was not significant.

When the pivaloyloxymethyl esters **12** and **14** were orally administrated in mice, the urinary recovery of **11** and **13** was determined by bioassay using *Escherichia coli* K-12 HW 8236 as a test strain after oral administration of the test samples (25 mg/kg as a parental cephalosporin) in mice ($n=3$, 0—4 h). The results are shown in Table II. The olefin geometry of **12** (*Z* isomer) and **14** (*E* isomer) had a significant effect on the urinary recovery in oral administration in mice. Compound **12** showed higher urinary recovery (21%) than **14** (15%) and was comparable with CFTM-PI. Therefore, ME1207 (**12**) was chosen as a candidate for further biological evaluation.

TABLE II. Urinary Recovery of Cephalosporin after Oral Administration in Mice (%)

Compound	ME1207 (**12**)	**14**	CFTM-PI	CFIX	CCL
Urinary recovery (%) (25 mg/kg, $n=3$, 0—4 h)	21.0	15.0	28.0	10.5	53.5

Clinical evaluation studies of ME1207 have been in progress.

Experimental

Melting points were uncorrected. Infrared (IR) spectra were recorded on a JASCO-IR-1 spectrometer. ^{1}H-NMR spcetra were determined with tetramethylsilane as an internal standard on either a Hitachi R-90H or JAXC 400GX, with chemical shifts given in ppm units. Mass spectra (MS) measurements were taken on a Hitachi M-80B mass spectrometer.

***p*-Methoxybenzyl 7-Phenylacetamido-3-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (4 and 5)** To the solution of *p*-methoxybenzyl 7-phenylacetamido-3-chloromethyl-3-cephem-4-carboxylate (10 g) in acetone (200 ml) were added PPh_3 (5.65 g) and NaI (3.2 g). The mixture was stirred at room temperature for 2 h and evaporated *in vacuo*. The residue was dissolved in dichloromethane (100 ml) and to the solution, 5-formyl-4-methylthiazole (**3**, 26.07 g) and 7% aq. sodium bicarbonate solution (100 ml) were added. After the mixture was stirred at room temperature for 17 h, the organic layer was washed with 10% aq. sodium hydrogen sulfite solution and brine, dried over $MgSO_4$ and evaporated *in vacuo*. The remaining residue was triturated in methanol (200 ml) to give a yellow crystal (**5**, 1.20 g) of *E* isomer. The filtrate was evaporated *in vacuo* and the remaining residue was purified by column chromatography on silica gel using benzene–ethyl acetate (5:1) as an eluent to give a pale yellow powder (**4**, 7.8 g) of *Z* isomer. **4** (*Z* isomer). mp 78—82 °C (dec.). IR (Nujol): 3200—3350, 1790, 1730, 1670, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 2.39 (3H, s, CH_3), 3.15, 3.45 (2H, ABq, *J*=16 Hz, 2-H), 3.62 (2H, s, CH_2), 3.77 (3H, s, OCH_3), 5.00 (1H, d, *J*=5 Hz, 6-H), 5.08 (2H, s, CH_2), 5.82 (1H, dd, *J*=5, 8 Hz, 7-H), 6.15 (1H, d, *J*=8 Hz, CONH), 6.40 (2H, d, *J*=14 Hz, arom), 6.80 (1H, d, *J*=11 Hz, CH=), 7.1—7.3 (8H, m, CH=, arom), 8.52 (1H, s, thiazole 2-H). Field desorption–mass spectra (FD-MS) *m/z*: 561 (M^+). **5** (*E* isomer). mp 174—175 °C (CH_2Cl_2). IR (Nujol): 3280, 1780, 1710, 1650, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 2.40 (3H, s, CH_3), 3.60 (2H, br s, 2-H), 3.62 (2H, s, CH_2), 3.78 (3H, s, OCH_3), 4.93 (1H, d, *J*=5 Hz, 6-H), 5.20 (2H, s, CH_2), 5.79 (1H, dd, *J*=5, 9 Hz, 7-H), 6.6—6.9 (4H, m, CH=, CONH, arom), 7.0—7.4 (8H, m, CH=, arom), 8.51 (1H, s, thiazole 2-H). FD-MS *m/z*: 562 $(M+H)^+$.

***p*-Methoxybenzyl 7-Amino-3(*Z*)-(4-methylthiazol-5-yl)-3-cephem-4-carboxylate (6)** To a solution of pyridine (1.04 ml) and phosphorus pentachloride (800 mg) in dichloromethane (20 ml), a solution of **4** (720 mg) in dichloromethane (3 ml) was added at −30 °C and the mixture was stirred at 0—5 °C for 2 h. The reaction mixture was poured into methanol (20 ml) at −20 °C and stirred for 1 h at 0—5 °C. The mixture was partitioned between dichloromethane (40 ml) and brine (20 ml) under ice-cooling, adjusted to pH 1.5—2.0 with 7% aq. sodium bicarbonate solution and stirred for 1 h at 0—5 °C. The separated organic layer was washed with brine and sat. $NaHCO_3$ dried over $MgSO_4$ and evaporated *in vacuo*. The remaining residue was purified by chromatography on silica gel using benzene–ethyl acetate (3:1) as an eluent and crystallized from ethyl acetate to give pale yellow crystals (443 mg) of **6**. mp 141—142 °C (ethyl acetate–dichloromethane). IR (Nujol): 1780, 1730, 1650, 1635, 1615 cm^{-1}. NMR ($CDCl_3$) δ: 2.40 (3H, s, CH_3), 3.20, 3.42 (2H, ABq, *J*=16 Hz, 2-H), 3.76 (3H, s, OCH_3), 4.75 (1H, d, *J*=5 Hz, 6-H), 5.00 (1H, d, *J*=5 Hz, 7-H), 5.08 (2H, s, CH_2), 6.25 (1H, d, *J*=11 Hz, CH=), 6.52 (1H, d, *J*=11 Hz, CH=), 6.76 (2H, d, *J*=8 Hz, arom), 7.18 (2H, d, *J*=8 Hz, arom), 8.52 (1H, s, thiazole 2-H). FD-MS *m/z*: 443 (M^+). *Anal.* Calcd for $C_{21}H_{21}N_3O_4S_2$: C, 56.87; H, 4.77; N, 9.47. Found: C, 56.81; H, 4.75; N, 9.31.

***p*-Methoxybenzyl 7-Amino-3(*E*)-(4-methylthiazol-5-yl)-3-cephem-4-carboxylate (7)** Using the procedure described for the preparation of **6**, this compound was prepared from **5**. Yellow crystals. mp 159—160 °C (ethyl acetate). IR (Nujol): 3420, 1780, 1720, 1610 cm^{-1}. NMR ($CDCl_3$) δ: 2.48 (3H, s, CH_3), 3.63, 3.71 (2H, ABq, *J*=18 Hz, 2-H), 3.80 (3H, s, OCH_3), 4.75 (1H, d, *J*=5 Hz, 6-H), 4.96 (1H, d, *J*=5 Hz, 7-H), 5.24, 5.28 (2H, ABq, *J*=12 Hz, CH_2), 6.83 (1H, d, *J*=16 Hz, CH=), 6.90 (2H, d, *J*=8 Hz arom), 7.27 (1H, d, *J*=16 Hz, CH=), 7.38 (2H, d, *J*=8 Hz arom), 8.57 (1H, s, thiazole 2-H). FD-MS *m/z*: 443 (M^+). *Anal.* Calcd for $C_{21}H_{21}N_3O_4S_2$: C, 56.87, H, 4.77; N, 9.47. Found: C, 56.58; H, 4.77; N, 9.28.

***p*-Methoxybenzyl 7-[(*Z*)-2-(2-Tritylaminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (9)** A solution of $POCl_3$ (214 mg) in dichloromethane (3 ml) was added dropwise to a solution of **6** (443 mg) and (*Z*)-2-(tritylaminothiazol-4-yl)-2-methoxyiminoacetic acid (**8**, 457 mg) in dichloromethane (30 ml) containing pyridine (0.32 ml) at −20 °C. After stirring for 2 h at −20 to −10 °C, the reaction mixture was poured into water (10 ml). The separated organic layer was washed with water and brine, dried over $MgSO_4$ and evaporated *in vacuo*. The remaining residue was purified by column chromatography on silica gel using benzene–ethyl acetate (5:1) as an eluent to give pale yellow powder (632 mg) of **9**. mp 134—136 °C (dec.). IR (Nujol): 3350, 1790, 1730, 1680, 1630, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 2.41 (3H, s, CH_3) 3.30, 3.48 (2H, ABq, *J*=18 Hz, 2-H), 3.78 (3H, s, OCH_3), 4.06 (3H, s, OCH_3), 5.08—5.15 (3H, m, 6-H, CH_2), 5.95 (1H, dd, *J*=5, 8.8 Hz, 7-H), 6.30 (1H, d, *J*=11.7 Hz, CH=), 6.58 (1H, d, *J*=11.7 Hz, CH=), 6.70 (1H, s, thiazole 5-H), 6.82 (2H, d, *J*=8 Hz, arom), 6.90 (1H, d, *J*=8.8 Hz, CONH), 7.03 (1H, br s, NH), 7.12—7.32 (17H, m, arom), 8.58 (1H, s, thiazole 2-H). FD-MS *m/z*: 869 $(M+H)^+$.

***p*-Methoxybenzyl 7-[(*Z*)-2-(2-Tritylaminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*E*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (10)** Using the procedure described for the preparation of **9**, this compound was prepared from **7** and **8**. A white powder. mp 137—138 °C (dec.). IR (Nujol): 3350, 1790, 1730, 1680, 1630, 1610 cm^{-1}. NMR ($CDCl_3$) δ: 2.47 (3H, s, CH_3), 3.62, 3.75 (2H, ABq, *J*=18 Hz, 2-H), 3.80 (3H, s, OCH_3), 4.07 (3H, s, OCH_3), 5.06 (1H, d, *J*=5 Hz, 6-H), 5.24 (2H, s, CH_2), 5.90 (1H, dd, *J*=5, 9 Hz, 7-H), 6.72 (1H, s, thiazole 5-H), 6.85 (1H, d, *J*=16 Hz, CH=), 6.90 (2H, d, *J*=8 Hz, arom), 7.00 (1H, d, *J*=9 Hz, CONH), 7.02 (1H, s, NH), 7.25—7.38 (18H, m, CH=, arom), 8.57 (1H, s, thiazole 2-H). FD-MS *m/z*: 869 $(M+H)^+$.

7-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylic Acid (11, ME1206) Compound **9** (200 mg) was treated with anisole (0.5 ml) and CF_3COOH (2 ml) at 0 °C for 1 h. The solution was diluted with isopropyl ether and the precipitate was triturated in isopropyl ether (100 ml). The resulting powder was dissolved in a mixture of water (1 ml) and ethyl acetate (3 ml) and the mixture was adjusted to pH 7.2 with $NaHCO_3$. The aqueous layer was chromatographed on a column of Diaion HP-20 using water–acetone (4:1) as an eluent. The fractions were collected and lyophilized to give the sodium salt (95 mg) of **11** as pale yellow crystals, which were recrystallized from water. mp 195—200 °C (dec.). IR (Nujol): 3450, 1775, 1680, 1620, 1590 cm^{-1}. NMR (dimethylsulfoxide (DMSO)-d_6) δ: 2.30 (3H, s, CH_3), 3.00, 3.28 (2H, ABq, *J*=18 Hz, 2-H), 3.82 (3H, s, OCH_3), 5.10 (1H, d, *J*=5 Hz, 6-H), 5.62 (1H, dd, *J*=5, 8 Hz, 7-H), 6.34 (1H, d, *J*=11 Hz, CH=), 6.71 (1H, s, thiazole 5-H), 6.77 (1H, d, *J*=11 Hz, CH=), 7.22 (2H, br s, NH_2), 8.89 (1H, s, thiazole 2-H), 9.54 (1H, d, *J*=8 Hz, CONH). Secondary ion mass spectrometer (SI-MS) *m/z*: 529 $(M+H)^+$. *Anal.* Calcd for $C_{19}H_{17}O_5N_3NaS_2 \cdot 1.5\ H_2O$: C, 41.07; H, 3.63; N, 15.13. Found: C, 41.2; H, 3.6; N, 15.2.

7-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*E*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylic Acid (13) Using the procedure described for the preparation of **11**, the sodium salt of **13** was prepared from **10**. A white powder. mp 184—185 °C (dec.). IR (Nujol): 3450, 1775, 1680 cm^{-1}. NMR (D_2O DOH at 4.82) δ: 2.50 (3H, s, CH_3), 3.86 (2H, br s, 2-H), 4.06 (3H, s, OCH_3), 5.34 (1H, d, *J*=5 Hz, 6-H), 5.87 (1H, d, *J*=5 Hz, 7-H), 6.97 (1H, d, *J*=16 Hz, CH=), 7.09 (1H, d, *J*=16 Hz, CH=), 7.08 (1H, s, thiazole 5-H), 8.77 (1H, s, thiazole 2-H). SI-MS *m/z*: 529 $(M+H)^+$. *Anal.* Calcd for $C_{19}H_{17}O_5N_3NaS_2 \cdot 2H_2O$: C, 40.42; H, 3.74; N, 14.89. Found: C, 40.99; H, 3.6; N, 14.62.

Pivaloyloxymethyl 7-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*Z*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (12, ME1207) A solution of **11** (30 mg) in DMF (3 ml) was treated with iodomethyl pivalate (95 mg) in DMF (1 ml) at −20 °C, and the mixture was stirred for 1 h at −20 °C. The reaction mixture was poured into cold water (20 ml) and extracted with ethyl acetate (20 ml). The organic layer was washed water (10 ml) and brine (10 ml), dried, and evaporated *in vacuo*. The remaining residue was purified by column chromatography on silica gel using ethyl acetate as an eluent to give a pale yellow powder (25 mg) of **12**. mp 127—129 °C. IR (Nujol): 3450, 1790, 1760, 1680, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 1.15 (9H, s, $C(CH_3)_3$), 2.44 (3H, s, CH_3), 3.30, 3.53 (2H, ABq, *J*=18.7 Hz, 2-H), 4.03 (3H, s, OCH_3), 5.21 (1H, d, *J*=5 Hz, 6-H), 5.52 (2H, br s, NH_2), 5.79, 5.85 (2H, ABq, *J*=5.5 Hz, CH_2), 6.11 (1H, dd, *J*=5, 8 Hz, 7-H), 6.37 (1H, d, *J*=11.7 Hz, CH=), 6.67 (1H, d, *J*=11.7 Hz, CH=), 6.80 (1H, s, thiazole 5-H), 7.93 (1H, d, *J*=8 Hz, CONH), 8.58 (1H, s, thiazole 2-H). SI-MS *m/z*: 621 $(M+H)^+$.

Pivaloyloxymethyl 7-[(*Z*)-2-(2-Aminothiazol-4-yl)-2-methoxyiminoacetamido]-3(*E*)-(4-methylthiazol-5-yl)vinyl-3-cephem-4-carboxylate (14) Using the procedure described for the preparation of **12**, this compound was prepared from **13**. A pale yellow powder. mp 128—130 °C. IR (Nujol): 3450, 1790, 1760, 1680, 1620 cm^{-1}. NMR ($CDCl_3$) δ: 1.21 (9H, s, $C(CH_3)_3$), 2.48 (3H, s, CH_3), 3.68, 3.78 (2H, ABq, *J*=18 Hz, 2-H), 4.05 (3H, s, OCH_3), 5.12 (1H, d, *J*=5 Hz, 6-H), 5.89 (2H, s, CH_2), 5.97 (1H, dd, *J*=5, 9 Hz, 7-H), 6.86 (1H, s, thiazole 5-H), 6.98 (1H, d, *J*=16 Hz, CH=), 7.33 (1H, d, *J*=16 Hz, CH=), 7.52 (1H, d, *J*=9 Hz, CONH), 8.57 (1H, s, thiazole 2-H). SI-MS *m/z*: 621 $(M+H)^+$.

Biological Evaluation MICs (μg/ml) were determined by the twofold agar dilution method using Sensitivity disk agar (Nissui Seiyaku, Co., Ltd.) after incubation at 37 °C for 20 h at inoculum sizes of 10^6 cfu/ml.

Urinary excretion was tested using male mice (Jcl:ICR, 4 weeks old). The test compounds were administered orally to three mice at a dose 25 mg/kg as a parental cephalosporin. Urinary recover rates (%) were calculated from the drug concentrations in urine at 0 to 4 h after

administration. Concentrations were determined by bioassay using *Escherichia coli* K-12 HW8236 as a test organism.

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A New Ionone Glucoside and a New Phenylpropanoid Rhamnoside from Stems of *Ampelopsis brevipedunculata* (MAXIM.) TRAUTV.[1)]

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A new ionone glucoside and a new phenylpropanoid rhamnoside, termed ampelopsisionoside (1) and ampelopsisrhamnoside (2), respectively, have been isolated from stems of *Ampelopsis brevipedunculata* (MAXIM.) TRAUTV. (Vitaceae) and their structures have been established based on lines of chemical and spectral evidence. Four known glycosides, tachioside (3), isotachioside (4), lyoniside (5), and 2-phenylethyl D-rutinoside (6), were also isolated.

Keywords *Ampelopsis brevipedunculata*; Vitaceae; stem; ionone glucoside; ampelopsisionoside; phenylpropanoid rhamnoside; ampelopsisrhamnoside

Leaves and roots of *Ampelopsis brevipedunculata* (MAXIM.) TRAUTV. (nobudou in Japanese) (Vitaceae) are used to prepare the Chinese crude drugs named She-pu-tao and She-pu-tao-gen, respectively, which are employed in China as remedies for nephritis and rheumatism, and as an external hemostatic for traumatic bleeding, *etc.*[2)] So far, flavonol glycosides,[3a,b)] and *p*-coumaric and caffeic acid derivatives,[3b)] have been isolated from leaves of *A. brevipedunculata*, and oligostilbenes[4)] from roots of *A. brevipedunculata* var. *hancei* REHDER. As part of our studies on the constituents of Vitaceae, we have now examined stems of *A. brevipedunculata*. This paper deals with the structure elucidation of a new ionone glucoside and a new phenylpropanoid rhamnoside, and identifications of four known glycosides.

After chromatographic and high-performance liquid chromatographic (HPLC) separation of the *n*-BuOH soluble part of the MeOH extract, two new glycosides, termed ampelopsisionoside (1) and ampelopsisrhamnoside (2), have been isolated together with four known glycosides, *i.e.*, tachioside (3),[5)] isotachioside (4),[5,6)] lyoniside (5),[7)] and 2-phenylethyl D-rutinoside (6).[8)]

Ampelopsisionoside (1), a white powder, $[\alpha]_D$ −35.1° (MeOH) showed carbonyl absorption at 1720 cm^{-1} in the infrared (IR) spectrum and had the molecular formula $C_{19}H_{32}O_8$ based on the $[M-H]^-$ peak at *m/z* 387 in the negative ion fast atom bombardment mass spectrum (FAB-MS). The electron impact mass spectrum (EI-MS) of 1 showed no molecular ion peak but gave two significant fragment ions at *m/z* 226 $[M^+-162$ (hexose unit)] and 208

1 : R = β-D-glucopyranosyl; 2 : R = α-L-rhamnopyranosyl; 7 : R = α-L-rhamnopyranosyl

Chart 1

TABLE I. ^{1}H-NMR (400 MHz) Data for 1[a)]

	1[b)]	1[c)]
2α-H	3.14, d, 13.3	2.87, d, 13.4
2β-H	2.05, dd, 13.3, 2.0	1.81, dd, 13.4, 2.1
4α-H	2.67, t, 13.3	2.44, t, 13.4
4β-H	2.30, m[d)]	2.12, ddd, 13.4, 4.6, 2.1
5β-H	2.26, m[d)]	2.27, ddq, 13.4, 4.6, 6.4
7-H	5.86, dd, 15.9, 0.9	5.72, dd, 15.9, 0.9
8-H	6.37, dd, 15.9, 6.4	5.90, dd, 15.9, 6.4
9-H	4.75, quint,[e)] 6.4	4.44, quint,[e)] 6.4
10-H_3	1.41, d, 6.4	1.32, d, 6.4
11-H_3	1.16, s	0.99, s
12-H_3	0.96, s	0.93, s
13-H_3	1.01, d, 6.4	0.90, d, 6.4
6α-OH	5.62, s	—
1′-H	4.96, d, 7.6	4.35, d, 7.9
2′-H	4.04, dd, 8.7, 7.6	3.18, t, 7.9
3′-H	4.26, t, 8.7	3.35, t, 7.9
4′-H	4.20, t, 8.7	3.31, t, 7.9
5′-H	3.92, ddd, 8.7, 5.2, 2.4	3.23, ddd, 7.9, 5.3, 2.4
6′-H_2	4.33, dd, 11.6, 5.2	3.65, dd, 11.9, 5.3
	4.52, dd, 11.6, 2.4	3.84, dd, 11.9, 2.4

a) Chemical shifts are in δ-values from internal tetramethylsilane (TMS) and are followed by multiplicities and coupling constant (Hz). *b*) In pyridine-d_5. *c*) In MeOH-d_4. *d*) Both multiplicities and coupling constant were obscure due to partial overlap. *e*) Observed with fine splitting due to the long-range coupling (0.9 Hz) with 7-H.

TABLE II. ^{13}C-NMR Data for 1 (100.5 MHz, Pyridine-d_5, δ_C, ppm, from TMS)[a)]

	1		1
1-C	43.11 (s)	11-C	25.30 (q)[c)]
2-C	52.08 (t)	12-C	24.71 (q)[c)]
3-C	210.67 (s)	13-C	16.61 (q)
4-C	45.87 (t)	1′-C	102.82 (d)
5-C	36.86 (d)	2′-C	75.50 (d)
6-C	77.17 (s)	3′-C	78.70 (d)
7-C	133.41 (d)[b)]	4′-C	71.95 (d)
8-C	134.61 (d)[b)]	5′-C	78.39 (d)
9-C	76.31 (d)	6′-C	62.95 (t)
10-C	21.42 (q)		

a) Assignments and multiplicities (in parentheses) were made with the aid of INEPT experiments. *b, c*) Assignments may be interchanged in each column.

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$[M^+ - 162 - H_2O]$.

The proton nuclear magnetic resonance (^1H-NMR) spectrum of **1** (Table I, in pyridine-d_5) showed signals ascribed to two tertiary methyls [δ 0.96, and 1.16], two secondary methyls [δ 1.01 (d, J=6.4 Hz) and 1.41 (d, J=6.4 Hz)], two olefinic protons [δ 5.86 (dd, J=15.9, 0.9 Hz) and 6.37 (dd, J=15.9, 6.4 Hz)], a hydroxyl proton [δ 5.62 (s)], and an anomeric proton [δ 4.96 (d, J=7.6 Hz)]. On methanolysis, **1** afforded methylglucoside as the sugar moiety. The results suggest that **1** is an ionone monoglucoside. The detailed ^1H- and carbon-13 nuclear magnetic resonance (^{13}C-NMR) assignments (Tables I and II, respectively) were made with the aid of ^1H–^1H correlation spectroscopy (^1H–^1H COSY), nuclear Overhauser effect correlation spectroscopy (NOESY), and insentive nuclei enhanced by polarization transfer (INEPT) techniques and it was inferred that **1** is an ionone glucoside with the relative structure **1** or its antipode, except for the configuration of the 9-OH group on the side chain.

The circular dichroism (CD) behavior of **1** showed a positive Cotton effect ([θ] +112) at 295 nm (MeOH) and the absolute stereochemistry of **1** was shown to be 5*R* (and 6*S*).[9] The structure of **1** was finally established as follows. The β-D-glucopyranosyl[10] moiety in **1** was corroborated by ^1H-NMR [the anomeric proton signal with a large coupling constant (J=7.6 Hz)] (Table I) and ^{13}C-NMR date (Table II).

TABLE III. ^1H-NMR (400 MHz) Data for **2** (MeOH-d_4)[a)]

	2
2-H	6.84, d, 2.1
5-H	7.00, d, 8.2
6-H	6.73, dd, 8.2, 2.1
7-H_2	2.66, m
8-H_2	1.89, m
9-H_2	3.36, 3.65, each 1H, m
OCH_3	3.84, s
1′-H_2 3′-H_2	3.74, 3.76, each 2H, each d, 5.0
2′-H	4.16, quint, 5.0
1″-H	4.64, d, 1.5
2″-H	3.79, dd, 3.4, 1.5
3″-H	3.65, m
4″-H	3.36, t, 9.5
5″-H	3.57, dd, 9.5, 6.4
6″-H_3	1.23, d, 6.4

a) Chemical shifts are in δ-values from TMS and are followed by multiplicities and coupling constant (Hz).

TABLE IV. ^{13}C-NMR Data for **2** (100.5 MHz, MeOH-d_4, δ_C, ppm, from TMS)[a)]

	2		**2**
1-C	138.05 (s)	1′-C	62.16 (t)
2-C	114.21 (d)	2′-C	83.41 (d)
3-C	152.09 (s)	3′-C	62.16 (t)
4-C	152.09 (s)	1″-C	101.82 (d)
5-C	119.61 (d)	2″-C	72.59 (d)[c)]
6-C	122.01 (d)	3″-C	72.43 (d)[c)]
7-C	32.47 (t)[b)]	4″-C	74.09 (d)
8-C	33.08 (t)[b)]	5″-C	69.85 (d)
9-C	67.79 (t)	6″-C	18.03 (q)
OCH_3	56.59 (q)		

a) Assignments and multiplicities (in parentheses) were made with the aid of INEPT experiments. *b*, *c*) Assignments may be interchanged in each column.

The position where the glucosyl residue is connected was made clear on the basis of the following NOESY experiments. A significant NOE cross peak between the anomeric proton of glucose (1′-H) and 9-H of the aglycone part was observed. This finding suggests that glucose is connected to the hydroxyl group at 9-C through a glycosidic linkage.[11] Based on the combined evidence, the structure of ampelopsisionoside is now established as **1**.[14]

Ampelopsisrhamnoside (**2**), colorless oil, $[\alpha]_D$ −18.1° (MeOH) had the molecular formula $C_{19}H_{30}O_9$ from high-resolution mass spectroscopic (HRMS) data (see Experimental). In addition, **2** gave two significant fragment ions at *m/z* 328 $[M^+ - 74$ (glycerol unit)] and *m/z* 255 $[M^+ - H - 146$ (deoxyhexose unit)].

The ^1H-NMR spectrum of **2** (Table III) showed signals due to three aromatic protons [δ 6.73 (dd, J=8.2, 2.1 Hz), 6.84 (d, J=2.1 Hz) and 7.00 (d, J=8.2 Hz)], one methoxy group [δ 3.84 (s)], an anomeric proton [δ 4.64 (d, J=1.5 Hz)], and a secondary methyl [δ 1.23 (d, J=6.4 Hz)]. The detailed ^1H- and ^{13}C-NMR assignments (Tables III and IV, respectively) were made with the aid of ^1H–^1H COSY, NOESY, and INEPT techniques and it was suggested that **2** consists of three groups, *i.e.*, a glycerol part [^1H-NMR δ 3.74, 3.76 (2H each, each d, J=5.0 Hz) (1′-H_2 and 3′-H_2) or (3′-H_2 and 1′-H_2), 4.16 (quint, J=5.0 Hz) (2′-H); ^{13}C-NMR δ 62.16 (1′-C and 3′-C), 83.41 (2′-C)], a phenylpropanoid part, and an α-L-rhamnopyranosyl residue.[15] The connections between these three groups were determined as follows. In the NOESY experiments on **2**, significant NOE cross peaks between 2′-H of the glycerol and 5-H of the phenylpropanoid unit, and between the anomeric proton of rhamnose and 9-H_2 of the phenylpropanoid unit were respectively observed. This finding indicates that glycerol and rhamnose are connected to the hydroxyl group at 4-C and the hydroxyl group at 9-C of the phenylpropanoid unit through ether and glycosidic linkages, respectively. Further, the ^1H- and ^{13}C-NMR data of **2** were analogous to those of compound **7**.[16] It was concluded that the 1-(3-methoxy-4-hydroxyphenyl)-glycerol unit in **7** was replaced by the glycerol unit in **2**. Based on the combined evidence, the structure of ampelopsisrhamnoside is established as **2**.[17]

Experimental

The instruments used to obtain melting points, optical rotations, IR, ^1H-NMR (400 MHz), ^{13}C-NMR (100.5 MHz), mass spectrum (MS), and gas liquid chromatography (GLC) data were the same as described in our preceding paper.[19] The CD spectrum was measured with a JASCO J-500 spectropolarimeter. Melting points are uncorrected. MS data were obtained under the following conditions (EI-MS and HRMS: ionization voltage, 30 eV. Negative ion FAB-MS: accelerating voltage, 2—3 kV; matrix, glycerol; collision gas, Xe). Optical rotations and CD spectra were determined for solutions in MeOH. GLC was carried out under the following operating conditions: column, 1.5% SE-52 on Chromosorb WAW DMCS (2 m × 3 mm i.d.); FID detector; column temperature, 180 °C; carrier gas, N_2 30 ml/min. For column chromatography, Merck Kieselgel 60 (230—400 mesh) and Sephadex LH-20 were used and for thin layer chromatography, precoated silica gel plates, Merck HF-254, were used. Preparative HPLC was carried out on a Waters instrument with a M 6000A pump, a U6K septumless injector, a series R-401 differential refractometer and a reversed phase ODS column (Waters μ-Bondapack-C_{18}; 7.8 mm × 30 cm) with H_2O–MeOH or H_2O–CH_3CN as eluents.

Plant Material Stems of *A. brevipedunculata* were collected at the campus of Setsunan University (Faculty of Pharmaceutical Sciences, Hirakata, Osaka, Japan) in October 1988 and identified by one of us (H.M.). A

voucher specimen is deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Setsunan University.

Extraction and Isolation of 1—6 The air-dried stems (2.8 kg) were extracted twice with MeOH (total 50 l) under reflux for 6 h. The combined extract was concentrated to dryness to afford the residue (550 g). A portion (440 g) of it was suspended in H_2O and the aqueous suspension was extracted successively with AcOEt (500 ml × 4), and *n*-BuOH (400 ml × 4). The residue (52.0 g) obtained from the *n*-BuOH layer was subjected to LH-20 column chromatography [LH-20 (500 ml); eluent MeOH] and a fraction (18.9 g) containing **1**—**6** was separated. This fraction was further purified by silical gel column chromatography ($CHCl_3$–MeOH as eluent) and repeated reversed-phase HPLC (H_2O–MeOH or H_2O–CH_3CN as eluents) to afford **1** (5.0 mg), **2** (2.6 mg), **3** (6.2 mg), **4** (8.3 mg), **5** (5.2 mg), and **6** (11.0 mg). The physical and spectral properties for **1**—**6** are as follows. Ampelopsisionoside (**1**), a white powder, $[\alpha]_D$ −35.1° (c=0.11). IR (KBr) cm^{-1}: 3375, 2910, 1720, 1070, 1030. EI-MS *m/z* (%): 226 (M^+ −162, 23), 208 (M^+ −162−H_2O, 63), 153 (95), 152 (94), 124 (100), 110 (63). Negative ion FAB-MS *m/z* (%): 387 {$[M(C_{19}H_{32}O_8)-H]^-$, 100}, 225 $[(M-H-162)^-$, 15]. ^1H- and ^{13}C-NMR: given in Tables I, and II, respectively. CD (c=0.092) $[\theta]_{20}$ (nm): +112 (295) (positive maximum). Ampelopsisrhamnoside (**2**), colorless oil, $[\alpha]_D$ −18.1° (c=0.04). IR (KBr) cm^{-1}: 3380, 2910, 1600, 1380, 1260, 1090, 1045. EI- and HRMS *m/z* (%): 402.190 (M^+, Calcd for $C_{19}H_{30}O_9$ 402.189, 100), 328.153 (M^+ −$C_3H_6O_2$, Calcd for $C_{16}H_{24}O_7$ 328.152, 7), 298 (11), 284 (29), 267 (9), 255.123 (M^+ −H−146, Calcd for $C_{13}H_{19}O_5$ 255.123, 56), 210 (90), 181 (72), 137 (93). Negative ion FAB-MS *m/z* (%): 401 $[(M-H)^-$, 100], 255 (19). ^1H- and ^{13}C-NMR: given in Tables III and IV, respectively. Tachioside (**3**),[5] mp 205—208 °C (ref. 5, mp 211—213 °C), $[\alpha]_D$ −58.0° (c=0.31) [ref. 5, −55.4° (MeOH)]. The melting point, optical rotation, IR, EI-MS, and ^1H-NMR data of **3** were consistent with the published data for tachioside.[5] Isotachioside (**4**),[5,6] mp 191—192 °C (ref. 5, mp 195—197 °C), $[\alpha]_D$ −44.8° (c=0.36) [ref. 5, −54.5° (MeOH)]. The melting point, optical rotation, IR, EI-MS, and ^1H-NMR data of **4** were consistent with the published data for isotachioside.[5,6] Lyoniside (**5**),[7] mp 157—159 °C (ref. 7*a*, mp 164—165 °C; ref. 7*f*, mp 162—164 °C), $[\alpha]_D$ +38.9° (c=0.20) [ref. 7*a*, +26.7° (MeOH); ref. 7*d*, +27.3° (MeOH)]. The melting point, optical rotation, IR, EI-MS, ^1H- and ^{13}C-NMR data of **5** were consistent with the published data for lyoniside.[7] 2-Phenylethyl D-rutinoside (**6**),[8] colorless amorphous solid, $[\alpha]_D$ −70.5° (c=0.55) [ref. 8, −101.2° (MeOH)]. Optical rotation, IR, EI- and negative FAB-MS, ^1H- and ^{13}C-NMR data of **6** were consistent with the published data for 2-phenylethyl D-rutinoside.[8]

Methanolysis of 1 A solution of **1** (1.0 mg) in 5% anhydrous HCl–MeOH (1 ml) was refluxed for 5 h. The reaction mixture was neutralized with Ag_2CO_3. The inorganic precipitate was filtered off and the filtrate was concentrated under reduced pressure to give the residue. The residue was trimethylsilylated with *N,O*-bis (trimethylsilyl)trifluoroacetamide-pyridine, and subjected to GLC analysis to demonstrate the presence of methyl glucoside.

Methanolysis of 2 A solution of **2** (0.5 mg) in 5% anhydrous HCl–MeOH (1 ml) was refluxed for 5 h. The reaction mixture was worked up in the same manner as in the case of **1** and subjected to GLC analysis to demonstrate the presence of methyl rhamnoside.

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9) The configurations of 13-H_3 and 6-OH in **1** were deduced as follows. In Table I (in MeOH-d_4), observed J values between 5β-H and 4α-H [13.4 Hz (diaxial relation)] and between 5β-H and 4β-H [4.6 Hz (axial–equatorial relation)] indicate the equatorial nature of 13-H_3. The configuration of 6-OH was deduced as follows. *a*) In Table I, it can be seen that the signals of 2α-H and 4α-H were shifted downfield by 6-OH (1,3-diaxial relation); *b*) In the NOESY experiment (in pyridine-d_5), correlation peaks between 6-OH and 11-H_3 and 13-H_3 were observed. Thus, α-equatorial and α-axial orientations of 13-H_3 and 6-OH were respectively assigned.
10) With respect to the configuration of glucose in **1**, the D form may be preferable from the viewpoint of natural occurrence.
11) All the signals due to the side chain carbons in **1** were similar in chemical shifts (and also multiplicities) to those of analogous 9-*O*-β-D-glucopyranosyl ionones, *i.e.*, roseoside,[12] and actinidioionoside.[13]
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14) It was not possible from our studies to determine the absolute configuration of the C_9 chiral center on the side chain.
15) With respect to the configuration of rhamnose in **2**, the L form may be preferable from the viewpoint of natural occurrence. Coupling constants $J_{1,2}$ (1.5 Hz) and $J_{2,3}$ (3.4 Hz) (Table III) may be reasonably assigned to diequatorial ($J_{1,2}$) and equatorial–axial ($J_{2,3}$) relations for α-anomeric orientation of L-rhamnose rather than axial–equatorial ($J_{1,2}$) and equatorial–axial ($J_{2,3}$) relations for β-anomeric orientation, because, in the latter orientation, it is assumed from inspection of a Dreiding model that $J_{1,2}$ and $J_{2,3}$ would have the same axial–equatorial relations, so that identical J-values would be expected.
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Phenolic Glycosides from Roots of *Adenophora tetraphylla* Collected in Heilongjiang, China

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***Adenophora tetraphylla* (= *A. triphylla*, Campanulaceae) is a source of the traditional Chinese medicine "Shashen". From the roots of this plant, three new phenolic glycosides, called shashenosides I, II and III (2—4) were isolated together with siringinoside (1), β-sitosteryl-β-D-glucoside, linoleic acid and methyl stearate. The common aglycone of 2, 3 and 4 was identified as 3-methoxy-5-(2′-propenyl)-1,2-benzenediol [= 1-*O*-methyl-5-(2′-propenyl)-pyrogallol = 6-hydroxyeugenol, 6], and the structures of 2 and 3 were elucidated as 2,3-di-*O*-β-glucopyranoside and 3-*O*-α-arabinopyranosyl-(1→6)-β-glucopyranoside of 6, respectively. Shashenoside III (4) was formulated as 2-*O*-β-glucopyranoside of 3. No saponin was found in the roots.**

Keywords *Adenophora tetraphylla*; Campanulaceae; Shashen; shashenoside; Chinese traditional medicine; synapyl-glycoside; 3-methoxy-5-(2′-propenyl)-1,2-benzenediol; glycoside

The traditional Chinese medicine, Shashen (沙参), roots of *Adenophora* spp. (Campanulaceae) is known to be an antiinflammatory and antitussive drug used in the treatment of lung disease. From *Adenophora* species, Konno *et al.* reported the isolation of sterols and triterpenoids[1] and Du *et al.* isolated triterpenoids and coumarins.[2] *A. tetraphylla* FISCH. *ex* JACKSON (= *A. triphylla* (THUNB.) A.DC.,[3] Chinese name: 輪葉沙参, 南参; Japanese name: saiyoushajin) is one of source plants of this crude drug. We have conducted studies on water soluble constituents of dangshen (党参), roots of *Codonopsis* spp. (Campanulaceae).[4—6] Continuing our chemical studies on campanulaceous traditional Chinese medicines, the present paper reports the isolation and structural elucidation of four phenolic glycosides from roots of *A. tetraphylla* collected in Heilongjian province, China.

As in the case of Dangshen, a methanolic extract of the roots contained a large amount of saccharides, which was removed by chromatography on highly porous synthetic polymer (Diaion HP-20). The resulting crude glycoside fraction was separated by combination of a variety of chromatography as described in the Experimental section to give four glycosides, **1**—**4** together with β-sitosteryl-β-D-glucoside, linoleic acid and methyl stearate.

Compound **1**, $C_{23}H_{34}O_{14}$ (from high resolution fast atom bombardment mass spectrometry (HR-FAB-MS)) afforded glucose on acid hydrolysis. Inspection of the negative FAB-MS as well as the ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectrum (in C_5D_5N) led to the identification of **1** as syringinoside (4-*O*-β-gentiobiosyl-3,5-dimethoxy-4-hydroxy-*trans*-cinnamylalcohol) which was recently isolated from *Wikstroemia sikokiana* FRANCH. *et* SAV. (Thymelaeaceae).[7]

Compound **2** called shashenoside I, $C_{22}O_{32}H_{13}$ (from HR-FAB-MS) afforded glucose and an aglycone (**6**), $C_{10}H_{12}O_3$ (from HR-FAB-MS) on acid hydrolysis. The ^{1}H-NMR spectrum of **6** (in CD_3OD) exhibited signals due to an allyl group at δ 3.27 (2H, dddd, J=6.8, 1.6, 1.3, 0.6 Hz, $-C\underline{H}_2-CH=CH_2$), 5.92 (1H, ddt, J=17.0, 10.1, 6.8 Hz, $-CH_2-C\underline{H}=CH_2$), 5.05 (1H, ddt, J=10.1, 2.0, 1.3 Hz, $-CH_2-CH=C\underline{H}_2$ (*cis*)), 5.08 (1H, ddt, J=17.0, 2.0, 1.6 Hz, $-CH_2-CH=C\underline{H}_2$ (*trans*)). Presence of an allyl group in **6** was also supported by the ^{13}C-NMR spectrum (in CD_3OD): signals at δ 40.1 ($-CH_2-$), 137.6 ($-CH=$), 115.7($=CH_2$). The ^{13}C-NMR (in CD_3OD) spectrum of **6** exhibited a methoxyl carbon signal at δ 56.2, four substituted aromatic carbon signals at δ 146.9, 143.9, 132.1, 130.7 and two unsubstituted aromatic carbon signals at δ 108.9, 103.5. The ^{1}H-NMR spectrum of **6** showed two hydroxy proton signals at δ 5.28 and 5.34 (each 1H, s), a methoxyl proton signal at δ 3.86

R		R¹	R²
1: -Glc⁶—Glc		**2**: -Glc	-Glc
5: -Glc		**3**: -Glc⁶—Ara	-H
		4: -Glc⁶—Ara	-Glc
		7: -Glc⁶—Ara	$-CH_3$

Glc: β-D-glucopyranosyl
Ara: α-L-arabinopyranosyl

Chart 1

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(3H, s) and a couple of *meta*-located aromatic proton signals at δ 6.30 (1H, d, J=1.8 Hz) and 6.44 (1H, dt, J=1.8, 0.6 Hz, long range coupling with methylene protons). The nuclear Overhauser effect (NOE) difference spectrum of **6** revealed the presence of NOE between two aromatic proton signals and the allylic methylene signal as well as between one of the aromatic proton signals (at δ 6.30) and the methoxyl proton signal (see Chart 1). These results led to assignment of the structure of **6** as 3-methoxy-5-(2′-propenyl)-1,2-benzenediol [=1-*O*-methyl-5-(2′-propenyl)-pyrogallol=6-hydroxyeugenol]. Compound **6** was used as a starting material for syntheses of natural phenolic compounds such as (±)-eusiderin,[8] but this is the first example of the occurrence of this compound in nature as an aglycone of glycosides.

The ^{1}H-NMR spectrum of **2** in CD_3OD exhibited two β-glucosyl anomeric proton signals at δ 4.79 (1H, d, J=7.9 Hz) and 5.80 (1H, d, J=7.9 Hz). The ^{13}C-NMR spectrum of **2** in C_5D_5N exhibited signals assignable to two sets of terminal β-glucopyranosyl units together with signals due to the aglycone carbons. Accordingly, **2** can be formulated as 1,2-di-*O*-β-glucopyranoside of **6**.

Compound **3**, called shashenoside II, $C_{21}H_{30}O_{12}$ (from HR-FAB-MS), yielded glucose, arabinose and the aglycone (**6**) on acid hydrolysis. The ^{1}H-NMR spectrum of **3** in CD_3OD showed two anomeric proton signals at δ 4.75 (1H, d, J=7.3 Hz) and 4.29 (1H, d, J=6.8 Hz). The ^{13}C-NMR spectrum of **3** in CD_3OD exhibited signals due to a terminal α-arabinopyranosyl unit and a 6-linked β-glucopyranosyl unit, indicating the presence of an α-arabinopyranosyl-(1→6)-β-glucopyranosyl moiety. In order to elucidate the location of the glycosyl moiety, a methyl ether (**7**) was prepared from **3** by treatment with diazomethane. As reported, in the ^{1}H-NMR spectra of **5**,[4] signals due to two aromatic and two methoxy protons appear at the same position, respectively, showing the symmetrical nature of the aromatic ring moiety. In contrast, the ^{1}H-NMR spectrum of **7** in CD_3OD exhibited signals due to the two aromatic and two methoxy proton signals at dfferent positions: at δ 6.66, 6.55 (each 1H, d, J=1.8 Hz, C-2,6), 3.81, 3.79 (each 3H, s, OCH_3), leading to the structure of **3** as 1-*O*-α-arabinopyranosyl-(1→6)-β-glucopyranoside of **6**. Presence of NOE between the signals at δ 3.81 and 6.55 as well as absence of NOE of another aromatic signal at δ 6.66 with both the methoxyl proton signals supported this formulation.

Compound **4**, called shashenoside III, $C_{27}H_{40}O_{17}$ (from HR-FAB-MS) gave glucose, arabinose and **6** on acid hydrolysis. The ^{1}H-NMR spectrum of **4** in CD_3OD exhibited three anomeric proton signals at δ 4.94 (1H, d, J=7.3 Hz), 4.89 (1H, d, J=6.3 Hz), 4.29 (1H, d, J=6.8 Hz). Comparison of the ^{13}C-NMR of **4** in CD_3OD with that of **3** revealed the presence of a terminal β-glucopyranosyl unit together with a α-arabinopyranosyl-(1→6)-β-glucopyranosyl moiety in **4**. On mild acid hydrolysis, **4** afforded **3**. It follows that **4** can be formulated as 1-*O*-β-glucopyranoside of **3**.

It has been mentioned that Shashen contains some saponins, and can be used as a substitute of Ginseng. However, in the present study, no saponin was isolated from the roots.

Experimental

General Procedures ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR spectra were recorded on a JEOL JNM GX-400 NMR spectrometer. FAB-MS was taken on a JEOL JMS SX-102 mass spectrometer by the direct inlet method. Preparative high performance liquid chromatography (HPLC) was carried out on a column of TSK-gel ODS-120T (21.5 mm i.d. × 30 cm): detection, ultraviolet (UV) at 254 nm; flow rate, 6 ml/min. Acid hydrolysis of glycosides and identification of the resulting monosaccharides by gas-liquid chromatography (GLC) were conducted in the usual manner.

Extraction and Separation Dried roots (8 kg) collected at Xiaoxinganling, Heilongjiang Province of China, were extracted with hot MeOH to give MeOH-extract (700 g). A suspension of the MeOH-extract (350 g) in H_2O was chromatographed on a column of Diaion HP-20 (Mitsubishi Kasei Co., Ltd.) with H_2O and then with MeOH. The H_2O-eluate was composed of a large amount of saccharides and other highly water soluble substances. Twenty grams of the MeOH-eluate (total: 40 g) was subjected to chromatography on silica gel with $CHCl_3$–MeOH–H_2O (30 : 10 : 1 then 14 : 6 : 1, homogenous) to give six fractions tentatively designated as fr. I—VI in their order of elution. Further chromatography of fr. I on silica gel with $CHCl_3$–MeOH (20 : 1) afforded β-sitosteryl 3-*O*-β-glucoside (150 mg) and linoleic acid (10 mg). Fraction II was chromatograhed on silica gel with $CHCl_3$–MeOH (6 : 1), affording methyl stearate. All of these compounds were identified by comparison of thin layer chromatography (TLC) and the ^{1}H- and ^{13}C-NMR spectra with corresponding authentic specimens.

Fractions II and III were respectively separated by HPLC on an octadecyl silica (ODS) column with 30% MeOH to give **3** (90 mg) from fr. III and **2** (228 mg) from fr. IV. Fractions V and VI were respectively subjected to chromatography on LiChroprep RP-18 (40—63 mμ, Merck) with 25% MeOH followed by HPLC on an ODS column with 24% MeOH, affording **1** (30 mg) from fr. V and **4** (40 mg) from fr. VI.

Syringinoside (**1**): A white powder, $[\alpha]_D^{18}$ −22.6° (c=0.31, MeOH), HR-FAB-MS (positive) m/z: Calcd for $[C_{23}H_{34}O_{14}+Na]^+$ 557.1846. Found 557.1884. FAB-MS m/z (negative): 533 $(M-H)^-$, 371 $(M-Glc-H)^-$, 209 $(M-Glc_2-H)^-$. ^{1}H-NMR in C_5D_5N: δ 6.89 (2H, s), 6.64 (1H, dt, J=16.1, 5.2 Hz), 6.86 (1H, d, J=16.1 Hz), 5.71 (1H, d, J=6.8 Hz), 4.93 (1H, d, J=7.8 Hz), 4.57 (2H, d, J=5.2 Hz), 3.83 (6H, s). ^{13}C-NMR in CD_3OD: aglycone carbons, δ 57.0 (2C), 63.6, 105.5, 130.0 (2C), 131.0, 131.3, 135.3, 154.3 (2C); sugar carbons, δ 62.8, 69.3, 71.4, 71.7, 75.2, 75.7, 77.9 (4C), 104.5, 105.1.

Shashenoside I (**2**): A pale yellow powder, $[\alpha]_D^{18}$ −47.6° (c=0.42, H_2O). HR-FAB-MS (positive) m/z: Calcd for $[C_{22}H_{32}O_{13}+Na]^+$ 527.1741. Found: 527.1780. FAB-MS (negative) m/z: 503 $(M-H)^-$, 341 $(M-Glc-H)^-$, 179 $(M-Glc_2-H)^-$. ^{1}H-NMR in CD_3OD: δ 3.84 (3H, s, OCH_3), 6.81, 6.60 (each 1H, d, J=1.8 Hz, a pair of *meta*-located aromatic protons) and allyl group signals at δ 3.29 (2H, ddd, J=6.4, 1.6, 1.3 Hz), 5.95 (1H, ddt, J=17.0, 10.1, 6.8 Hz), 5.05 (1H, ddt, J=10.1, 2.0, 1.3 Hz), 5.06 (1H, ddt, J=17.0, 2.0, 1.6 Hz). ^{13}C-NMR in CD_3OD: aglycone carbons, δ 41.2 (C-α), 57.0 (OCH_3), 109.3, 112.0 (C-6,2), 116.3 (C-γ), 135.5 (C-4), 138.5 (C-β), 138.7 (C-1), 152.4, 154.3 (C-3,5); sugar carbons, δ 62.5, 62.6 (C-6), 71.3, 71.4 (C-4), 75.1, 75.7 (C-2), 77.7, 78.0, 78.4 (2C) (C-3,5), 103.8, 105.5 (C-1).

A solution of **2** (45 mg) in a mixture of 2% HCl and 50% dioxan for 2 h at 75 °C. The reaction mixture was extracted with C_6H_6 and the benzene extract was chromatographed on silica gel with $CHCl_3$–MeOH (20 : 1) to give **6**: A yellow powder. HR-EI-MS m/z: Calcd for $C_{10}H_{12}O_3^+$ 180.0786. Found: 180.0766.

Shashenoside II (**3**): A yellow powder, $[\alpha]_D^{18}$ −51.4° (c=0.68, MeOH). HR-FAB-MS (positive) m/z: Calcd for $[C_{21}H_{30}O_{12}+Na]^+$ 497.1635. Found: 497.1651. ^{1}H-NMR in CD_3OD: δ 6.66, 6.54 (each 1H, d, J=1.8 Hz, C-2,4), 5.94 (1H, ddt, J=16.8, 10.1, 6.8 Hz, C-β), 5.06 (1H, dd, J=16.8, 1.8 Hz, C-γ*trans*), 5.03 (1H, ddt, J=10.1, 1.8, 1.0 Hz, C-γ*cis*), 4.75 (1H, d, J=7.3 Hz, anomeric H), 4.29 (1H, d, J=6.6 Hz, anomeric H), 3.82 (3H, s, OCH_3), 3.29 (2H, dd, J=6.8, 1.0 Hz, C-α). ^{13}C-NMR in CD_3OD: aglycone carbons, δ 40.9 (C-α), 56.8 (OCH_3), 108.9, 112.3 (C-6,2), 115.9 (C-γ), 132.5 (C-1), 136.0 (C-4), 139.1 (C-β), 146.7, 149.6 (C-5,3); anomeric carbons δ 104.3, 104.8; α-arabinoside carbons, δ 66.6 (C-5), 69.2 (C-4), 72.3 (C-2), 74.1 (C-3); β-glucoside carbons, δ 69.3 (C-6), 71.3 (C-4), 74.8 (C-2), 77.0 (C-5), 77.4 (C-3). On acid hydrolysis in the same way as **2**, **3** afforded glucose, arabinose and **6**. Shashenoside III (**3**) (15 mg) was treated with CH_2N_2 in Et_2O–MeOH and the product was purified by HPLC on an ODS column with 35% MeOH to give a methyl ether **7**: A yellow powder. HR-FAB-MS (positive) m/z: Calcd for $[C_{22}H_{32}O_{12}+Na]^+$ 511.1791. Found: 511.1820. ^{1}H-NMR in CD_3OD: δ 6.66 (1H, d, J=1.8

Hz, C-2), 6.55 (1H, d, J=1.8 Hz, C-6), 5.96 (1H, m, C-β), 5.10 (1H, dd, J=16.7, 2.0 Hz, C-γ*trans*), 5.04 (1H, dd, J=10.0, 2.0 Hz, C-γ*cis*), 4.94 (1H, d, J=7.0 Hz, anomeric H), 4.26 (1H, d, J=6.7 Hz, anomeric H), 3.81 (3H, s, 5-OCH_3), 3.79 (3H, s, 4-OCH_3), 3.29 (2H, d, J=7.3 Hz, C-α).

Shashenoside III (**4**): A yellow powder, $[\alpha]_D^{18}$ $-42.2°$ (c=0.45, MeOH). HR-FAB-MS (positive) m/z: Calcd for $[C_{27}H_{40}O_{17}+Na]^+$659.2163. Found: 659.2184. Calcd for $[C_{27}H_{40}O_{17}+K]^+$ 675.1903. Found: 675.1940. FAB-MS (negative) m/z: 635 $(M-H)^-$, 473 $(M-Glc-H)^-$, 341 $(M-Glc-Ara-H)^-$, 179 $(M-Glc_2-Ara-H)^-$. ^{1}H-NMR in CD_3OD: δ 6.77, 6.66 (each 1H, d, J=1.8 Hz, C-2,6), 5.96 (1H, m, C-β), 5.07 (2H, m, C-γ), 4.94 (1H, d, J=7.3 Hz, anomeric H), 4.89 (1H, d, J=6.3 Hz, anomeric H), 4.29 (1H, d, J=6.8 Hz, anomeric H), 3.82 (3H, s, OCH_3), 3.31 (2H, d, J=6.6 Hz, C-α). ^{13}C-NMR in CD_3OD: aglycone carbons, δ 41.2 (C-α), 57.2 (OCH_3), 109.4, 112.1 (C-2,6), 116.4 (C-γ), 135.6 (C-4), 138.6 (C-β), 138.7 (C-1), 152.0, 154.4 (C-3,5); anomeric carbons, δ 103.7, 105.0, 105.4; α-arabinoside carbons, δ 66.6 (C-5), 69.3 (C-4), 72.4 (C-2), 74.2 (C-3); β-glucoside carbons, δ 62.5 (C-6), 69.4 (C-6), 71.2 (C-4), 71.3 (C-4), 75.0 (C-2), 75.8 (C-2), 77.1, 77.4, 77.9, 78.3 (C-3,5). On acid hydrolysis in the same way as **2**, **4** gave glucose, arabinose and **6**.

A solution of **4** (15 mg) in 1% H_2SO_4 (20 ml) was heated at 70 °C for 3 h. The reaction mixture was neutralized with diluted NaOH solution and then extracted with 1-BuOH saturated with H_2O. The BuOH extract was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (30 : 10 : 1, homogeneous) to give **3** which was identified by comparison of the ^{1}H- and ^{13}C-NMR spectra, optical rotation and TLC with those of an authentic sample.

Acknowledgements We are grateful to the Japan Society for the Promotion of Science, Tokyo for the fellowship for C. H. Shao's study in Hiroshima University. Thanks are also due to Professor M. Kozawa, Osaka University of Pharmaceutical Sciences for his valuable advice.

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Labdane-Type Diterpene Glycosides from Fruits of *Rubus foliolosus*

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From fruits of *Rubus foliolosus* (Rosaceae), a traditional medicine used in Yunnan, China, seven labdane-type diterpene glycosides were isolated. Of these glycosides, five were identified as goshonosides-F1—5 (1—5) which have been isolated from leaves of *R. chingii*. Structures of two new glycosides, goshonosides-F6 (6) and -F7 (8), were elucidated as α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside of 13(*E*)-*ent*-labda-8(17),13-diene-3β,15,18-triol and 3β,15-di-*O*-β-D-glucopyranoside of 13(*E*)-*ent*-labda-8(17),13-diene-3β,15-diol. Goshonosides-F6 (6) and -F7 (8) were also isolated from the leaves of *R. chingii*.

Keywords Chinese traditional medicine; *Rubus foliolosus*; *Rubus chingii*; Rosaceae; diterpene glycoside; labdane type diterpene; goshonoside; fu-pen-zi

Previously, rubusoside (13,19-di-*O*-β-D-glucosyl-steviol), a sweet kaurane-type diterpene glycoside had been isolated from leaves and fruits of *Rubus suavissimus* S. LEE[1,2] which grows in Guang-xi and Guang-dong, southern China. Very recently, another sweet kaurane-type diterpene glucoside, named suavioside, has also been isolated from the leaves of this plant.[3]

In China and Korea, fruits of some of the *Rubus* spp. have been used as a tonic for aged people (覆盆子 Chinese name: fu-pen-zi; Korean name: bog-bun-ja). In relation to the chemical identification of the source plant of this crude drug, chemotaxonomical studies on a number of *Rubus* spp. growing in Eastern Asia have been conducted. *R. chingii* HU which grows in An-fui, Jiang-su, Zhe-jiang, Jiang-xi and Fu-jien, China and also in Yamaguchi, Ohita and Kochi, Japan (Japanese name: gosho-ichigo), is morphologically very similar to *R. suavissimus*. From the leaves of *R. chingii*, no rubusoside but several labdane type diterpene glycosides, named goshonosides-F1 (**1**), -F2 (**2**), -F3 (**3**), -F4 (**4**) and -F5 (**5**) were isolated in yields of 0.2, 0.2, 0.4, 0.5 and 5.7%, respectively,[4] all of which do not taste sweet (yields in the previous paper[4] are erroneous and must be amended as above). Goshonoside-F5 (**5**) was isolated from the fruits of this plant and also from commercial fu-pen-zi purchased in Kuang-zhou.[5] This indicates that the plant source of this drug in southern China is *R. chingii*.

We have further studied the glycosides of a number of *Rubus* spp. other than *R. suavissimus* and *R. chingii*. However, no diterpene glycoside has been isolated but several 19α-hydroxyursane-type triterpene glycosides have been isolated.[6] In continuing these serial studies, a characteristic dimeric triterpene glycoside, named coreanoside F1 was very recently isolated from the leaves and fruits of *R. coreanus* MIQ. which has been used as one of the plant sources of fu-pen-zi in Korea and northern China.[7] Coreanoside F1 was also isolated from commercial Korean bog-bun-ja but not identified in the leaves and fruits of *R. crataegifolius* BUNGE. and *R. parvifolius* LINN., both of which are also described as the plant sources of this crude drug. The present paper reports on the glycosides of the fruits of *R. foliolosus* D. DON. which is also used as fu-pen-zi in Yunnan, south-western China.

The fruits collected in Dali, Yunnan were extracted with methanol. A suspension of the methanolic extract in water was washed with ethyl acetate and then chromatographed as described in the Experimental, affording seven glycosides. Of these, five were identified as goshonosides-F1—5 (**1**—**5**) already obtained from *R. chingii*.

A new glycoside, **6** yielded D-glucose and L-arabinose on acid hydrolysis. It was revealed that signals assigned to the aglycone moiety of **2**[4] appeared at almost the same positions in the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum of **6**. This indicated that **6** must be a 18-*O*-glycoside of 13(*E*)-*ent*-labda-8(17),13-diene-3β,15,18-triol (**7**) which is the common aglycone of **1**, **2** and **5**. Furthermore, the ^{13}C-NMR spectrum of **6** exhibited signals due to a terminal α-arabinofuranoside unit[8] and a 6-linked β-glucopyranoside unit. It follows that **6** can be formulated as 18-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside of **7**.

Another new glycoside, **8** afforded D-glucose on acid hydrolysis. On hydrolysis with β-glucosidase, **8** yielded D-glucose and an aglycone which was identified as 13(*E*)-*ent*-labda-8(17),13-diene-3β,15-diol (**9**) by comparison of the optical rotation with literature[9] and the NMR with the corresponding enantiomeric compound which has been isolated from the leaves of *Acacia* spp.[10] The ^{13}C-NMR spectrum of **8** showed carbon signals due to two terminal β-D-glucopyranosyl units and the glucosylation shift[11] was observed for the signals due to carbons around C-3 and

	R^1	R^2	R^3
1	OH	Glc	CH_2OH
2	OH	H	CH_2O–Glc
3	H	Glc	COO–Glc
4	H	Glc	CH_2O–Glc
5	OH	Glc	CH_2O–Glc
6	OH	H	CH_2O–Glc-(6←1)-Ara*f*
7	OH	H	CH_2OH
8	O–Glc	Glc	CH_3
9	OH	H	CH_3

Glc: β-D-glucopyranosyl Ara*f*: α-L-arabinofuranosyl

Chart 1

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TABLE I. ^{13}C-NMR Data for Compounds **6**, **7**, **8** and **9** (δ from TMS in C_5D_5N)

	6	7	8	9
Aglycone				
C- 1	38.1	38.3	38.4	39.5
C- 2	27.8	28.3	24.5	27.8
C- 3	71.8	72.8	84.8	79.6
C- 4	43.4	43.3	38.9	40.1
C- 5	46.9	47.6	55.6	56.6
C- 6	24.3	24.3	24.4	24.3
C- 7	37.1	37.3	37.0	37.1
C- 8	148.9	148.8	148.6	148.6
C- 9	56.5	56.4	55.1	55.1
C-10	39.5	39.6	39.5	39.5
C-11	22.5	22.4	21.8	21.9
C-12	38.9	38.7	38.5	38.7
C-13	137.6	137.4	140.9	137.4
C-14	125.8	125.9	121.5	125.9
C-15	59.0	58.9	65.3	58.9
C-16	16.5	16.4	16.3	16.5
C-17	106.5	106.6	106.9	106.7
C-18	74.2	67.3	28.7	28.7
C-19	12.8	12.9	17.0	16.9
C-20	15.3	15.2	14.7	14.8
3-*O*-Glc				
1			102.5	
2			75.3	
3			78.4	
4			72.2	
5			78.7	
6			63.4	
15-*O*-Glc				
1			102.9	
2			75.2	
3			78.6	
4			71.8	
5			78.7	
6			62.9	
18-*O*-Glc				
1	105.5			
2	74.9			
3	78.4			
4	72.3			
5	76.7			
6	68.9			
Ara*f*				
1	110.2			
2	83.2			
3	78.5			
4	86.0			
5	62.7			

-15. Based on these results, **8** can be formulated as 3,15-di-*O*-β-D-glucopyranoside of **9**. Since **6** and **8**, were also isolated from the leaves of *R. chingii* in the present study, the names, goshonosides-F6 and -F7 were proposed for **6** and **8**, respectively. Both **6** and **8** were also detected in commercial fu-pen-zi purchased in guang-zhou.

The present study demonstrated that the chemical distinction between commercial fu-pen-zi prepared from the fruits of *R. foliolosus* and those from *R. chingii* is difficult by using glycosides as the marker. It is noteworthy that fruits of *R. foliolosus* can be readily morphologically distinguished from those of *R. chingii.*

Experimental

Melting points were uncorrected. Optical rotations were measured with a Union PM-101. ^{1}H-NMR (400 MHz, in C_5D_5N or $CDCl_3$) and ^{13}C-NMR (100 MHz, in C_5D_5N) spectra were run on a JEOL JMN-GX400 using tetramethylsilane as an internal standard. Acid hydrolysis of glycosides followed by identification of the resulting monosaccharide including absolute configuration,[12] and the methylation analysis of the sugar moieties monitored by gas chromatography–mass spectrometry (GC-MS) were carried out as described in the previous paper.[13]

Extraction and Isolation The dried fruit (500 g) of *Rubus foliolosus* D. DON, collected in Dali, Yunnan, China, was extracted with hot MeOH. The MeOH extract (32.8 g) was suspended in H_2O and defatted with EtOAc, and the aqueous layer was applied on a column of Diaion HP-20. The column was washed with H_2O and then eluted with 40% MeOH, 80% MeOH, MeOH and acetone. The 80% MeOH eluate (3.3 g) contained goshonosides, and this fraction was chromatographed on a Si-gel column using AcOEt–EtOH–H_2O [16:2:1, 8:2:1 and 5:2:1] to give seven fractions (frs. 1—7). Fraction 2 was purified by high performance liquid chromatography (HPLC) [TSKgel ODS-120T (21.5 mm × 30.0 cm), MeOH–H_2O (70:30)] to give **1** (0.0012%) and **2** (0.0014%). Fraction 3 was separated by HPLC [YMC-Pack ODS (20.0 mm × 25.0 cm), MeOH–H_2O (78:22)] to give **6**, **3**, **8** and **4** in yields of 0.0012, 0.0016, 0.0027 and 0.0014%, respectively. Fraction 5 was purified by HPLC [TSKgel ODS-120T (21.5 mm × 30.0 cm), MeOH–H_2O (65:35)] to give **5** (0.036%). **1**: Colorless prisms from MeCN–H_2O, mp 95—98 °C, $[\alpha]_D^{18}$ −55.6° (c=1.91, MeOH). **2**: A white powder, $[\alpha]_D^{18}$ −30.4° (c=0.96, MeOH). **3**: A white powder, $[\alpha]_D^{18}$ −31.5° (c=0.65, MeOH). **4**: A white powder, $[\alpha]_D^{18}$ −36.5° (c=0.75, MeOH). **5**: A white powder, $[\alpha]_D^{18}$ −45.2° (c=1.85, MeOH).

Goshonoside F6 (6) A white powder, $[\alpha]_D^{18}$ −28.4° (c=0.56, MeOH). *Anal.* Calcd for $C_{31}H_{52}O_{12} \cdot H_2O$: C, 58.66; H, 8.56. Found: C, 58.72; H, 8.52%. ^{1}H-NMR (in C_5D_5N) δ: 0.71 (3H, s, H_3-20), 0.88 (3H, s, H_3-19), 1.66 (3H, s, H_3-16), 3.53, 4.36 (each 1H, each d, J=9.8 Hz, H_2-18), 4.21 (1H, dd, J=4.4, 12.4 Hz, H-3), 4.47 (2H, br d, J=6.3 Hz, H_2-15), 4.57, 4.88 (each 1H, br s, H_2-17), 5.77 (1H, t, J=6.3 Hz, H-14), 4.84 (1H, d, J=7.8 Hz, H-1 of Glc), 5.69 (1H, d, J=2.0 Hz, H-1 of Ara). ^{13}C-NMR data is listed in Table I.

Enzymatic Hydrolysis of 6 Goshonoside F6 (**6**, 20 mg) was treated with crude hesperidinase at 40 °C for 16 h, and then extracted with EtOAc to give **7** (12 mg).

13(*E*)-*ent*-Labda-8(17),13-diene-3β,15,18-triol (7) Colorless prisms from $CHCl_3$, mp 141—143 °C, $[\alpha]_D^{18}$ −29.0° (c=0.98, $CHCl_3$). ^{1}H-NMR (in $CDCl_3$) δ: 0.73 (3H, s, H_3-20), 0.82 (3H, s, H_3-19), 1.66 (3H, s, H_3-16), 3.36, 3.65 (each 1H, each d, J=10.0 Hz, H_2-18), 3.65 (1H, dd, J=4.5, 12.0 Hz, H-3), 4.13 (2H, br d, J=6.3 Hz, H_2-15), 4.53, 4.85 (each 1H, each br s, H_2-17), 5.37 (1H, t, J=6.3 Hz, H-14). ^{13}C-NMR data is listed in Table I.

Goshonoside F7 (8) A white powder, $[\alpha]_D^{18}$ −47.7° (c=0.55, MeOH). *Anal.* Calcd for $C_{32}H_{54}O_{12}$: C, 60.93; H, 8.63%. Found: C, 61.01; H, 8.59%. ^{1}H-NMR (in C_5D_5N) δ: 0.69 (3H, s, H_3-20), 0.91 (3H, s, H_3-19), 1.26 (3H, s, H_3-18), 1.69 (3H, s, H_3-16), 3.69 (1H, dd, J=4.4, 11.7 Hz, H-3), 4.54 (1H, dd, J=7.8, 12.2 Hz, H_a-15), 4.61 (1H, d, J=5.3, 12.2 Hz, H_b-15), 4.60, 4.90 (each 1H, each br s, H_2-17), 5.58 (1H, dd, J=5.3, 7.8 Hz, H-14), 4.96 (1H, d, J=7.3 Hz, H-1 of 15-*O*-Glc), 4.99 (1H, d, J=7.8 Hz, H-1 of 3-*O*-Glc). ^{13}C-NMR data is listed in Table I.

Enzymatic Hydrolysis of 8 An aqueous solution (2 ml) of **8** (15 mg) was incubated with β-glucosidase (10 unit, from sweet almonds, Sigma) at 40 °C for 24 h, and then extracted with EtOAc to give **9** (10 mg).

13(*E*)-*ent*-Labda-8(17),13-diene-3β,15-diol (9) Colorless needles from $CHCl_3$, mp 161—163 °C, $[\alpha]_D^{18}$ −29° (c=0.56, $CHCl_3$). ^{1}H-NMR (in $CDCl_3$) δ: 0.69 (3H, s, H_3-20), 0.77 (3H, s, H_3-19), 0.99 (3H, s, H_3-18), 1.67 (3H, s, H_3-16), 3.35 (1H, dd, J=4.5, 12.1 Hz, H-3), 4.14 (2H, br d, J=7.1 Hz, H_2-15), 4.53, 4.85 (each 1H, each br s, H_2-17), 5.39 (1H, dd, J=5.3, 7.8 Hz, H-14). ^{13}C-NMR data is listed in Table I.

Acknowledgement This study was supported by a Grant-in-Aid (Monbusho International Scientific Program between Hiroshima University and Kunming Institute of Botany, Chinese Academy of Science, 1988—1989 and 1991—1993) from the Ministry of Education, Science and Culture to which our thanks are due.

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Chem. Pharm. Bull. 39(9) 2446—2448 (1991) Vol. 39, No. 9

Carbon-13 Nuclear Magnetic Resonance Studies on the Biosynthesis of Hyaluronic Acid

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The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of ^{13}C-labeled hyaluronic acid (HA, 1a) was measured to clarify the origin of the carbon atoms of HA. ^{13}C-Labeled HA (1a) was obtained from the cell surface of *Streptococcus zooepidemicus #104* after administration of [1-^{13}C]-D-glucose (2). The isolated HA, which had a molecular weight of more than one million, showed three signals in the ^{13}C-NMR spectrum with no chemical degradation. The results revealed that [1-^{13}C]-D-glucose (2) was incorporated into the [1-^{13}C]-D-glucuronic acid units and [1-^{13}C]-*N*-[^{13}CH$_3$]acetamido-glucosamine units of HA (1a). The predicted biosynthetic pathway of HA was confirmed by this experiment.

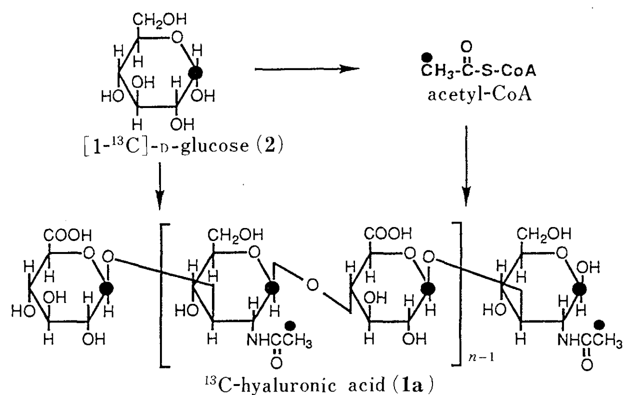
Keywords ^{13}C-NMR; hyaluronic acid; [1-^{13}C]-D-glucose; *Streptococcus zooepidemicus #104*; biosynthesis

Introduction

Hyaluronic acid (HA, **1**) is one of the acid mucopolysaccharides, having alternating β-1-3 glucuronic acid and β-1-4 glucosaminidic bonds.[1] HA (**1**) is present in the cell coat and in the extracellular ground substance of the connective tissues of vertebrates; it also occurs in the synovial fluid in joints and in the vitreous humor of the eye.[2] HA (**1**) has many physiological functions based on its physical characteristics of viscoelasticity and moisture retention, *i.e.*, it is involved in the control of electrolytes and water in extracellular fluids, protection against mechanical deformation, wound healing, protection against infection, maintenance of the stable transparent fluid in the eye, and control of cellular differentiation and multiplication.[3]

HA (**1**) has been applied in cosmetics as a moisturizer and has also found opthalmic, orthopedic, dermatological, and other applications in medicine. In the present work, we prepared ^{13}C-labeled HA (**1a**) for studies on the biosynthesis of HA (**1**) by ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy. A previous study by Roseman *et al.*[4] on HA (**1**) biosynthesis involved partial chemical degradation of ^{14}C-labeled HA (**1b**), but we wished to avoid possible errors arising from the use of chemical degradation methods.

Nuclear magnetic resonance (NMR) spectroscopy can give detailed information about molecular structure without the need for chemical degradation, particularly for compounds with a molecular weight of under 1500.[5] However, the molecular weight of HA (**1**) is within the range of 50000 to 8×10^6, depending on the source and method of preparation,[1,6] and so it is not possible to obtain a distinct and natural abundance of ^{13}C-NMR signals, although proton nuclear magnetic resonance (^{1}H-NMR)



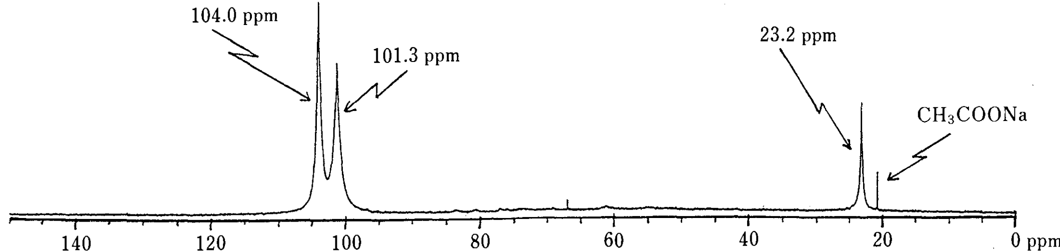


Fig. 1. The 125 MHz ^{13}C-NMR Spectrum of HA (**1a**) from [^{13}C]-D-Glucose (**2**)

In the structural formulas, ● = ^{13}C-enriched carbons. Solvent = D$_2$O; number of scans = 50000; acquisition time = 0.87 s; size of data table = 16 k points; pulse width = 45°. Shifts are relative to TSP.

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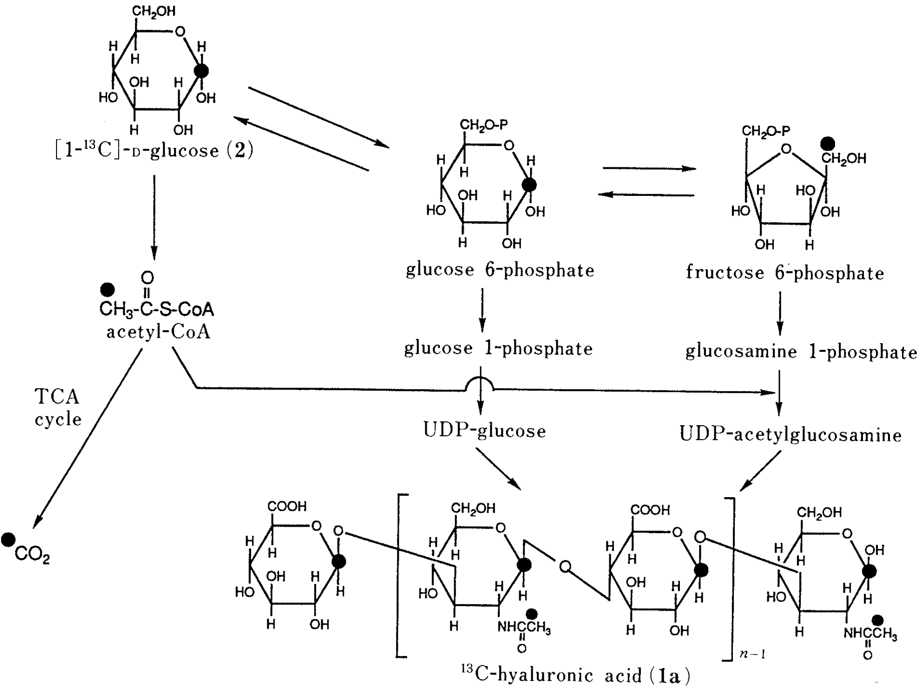


Fig. 2. Biosynthetic Pathway to HA (**1a**)

● = ^{13}C-enriched carbons.

signals have been measured.[7] We therefore prepared ^{13}C-labeled HA (**1a**) for examination of the biosynthesis of HA (**1**).

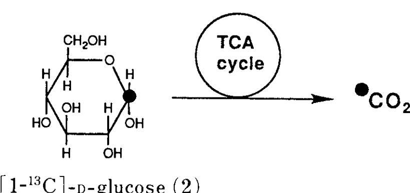
For measurement of the NMR spectrum, we required HA (**1**), in high purity, free from proteins or other mucopolypeptides. Since the preparation of HA (**1**) from animal tissue in high purity is difficult, we obtained HA (**1**) from *Streptococcus* (*S.*) *zooepidemicus #104*, which is an HA (**1**)-producing bacterium, in an established way.[8] [1-^{13}C]-D-Glucose (**2**) was fed to *S. zooepidemicus #104* and ^{13}C-labeled HA (**1a**) was isolated from the cell surface.

Further, the exhaust gas from a growing culture was bubbled into sodium hydroxide solution, and infrared (IR) and ^{13}C-NMR spectral measurements were made to confirm that ^{13}C-carbon dioxide was produced as a bacterial metabolite of [1-^{13}C]-D-glucose (**2**) by way of the tricarboxylic acid (TCA) cycle.[9] IR measurement is simpler and more convenient, compared with mass spectroscopy. The ^{13}C excess percent was calculated from the IR absorption intensities of $^{13}C=O$ ($2280 \pm 10\,cm^{-1}$) and $^{12}C=O$ ($2380 \pm 10\,cm^{-1}$).[10]

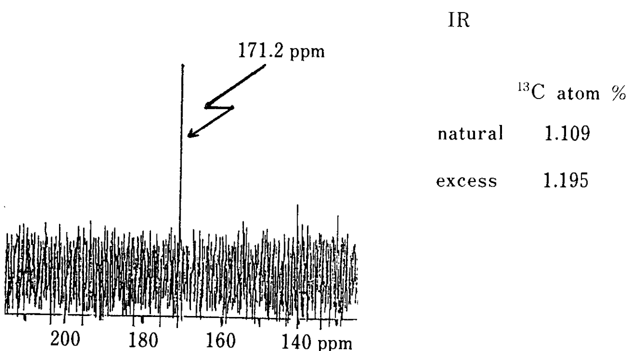
Results and Discussion

[1-^{13}C]-D-Glucose (99% atom ^{13}C: α-form, C-1 = 92.8 ppm; β-form, C-1 = 96.7 ppm) was fed to a culture of *S. zooepidemicus #104*, and HA (**1a**) was obtained, as described in Materials and Methods.

The ^{13}C-NMR spectrum of HA (**1a**) thus prepared exhibited three intense NMR signals (23.2, 101.3, 104.0 ppm), as shown in Fig. 1. The spectrum clearly showed ^{13}C enrichment of two carbons at 23.2 and 104.0 ppm (acetyl units and C-1 carbon of *N*-acetamide glucosamine units, respectively) in comparison with the natural abundance intensities. The 101.3 ppm signal was assigned to [1-^{13}C]-D-glucuronic acid units. These signals indicated that [1-^{13}C]-D-glucose (**2**) had been incorporated into HA (**1a**)



NMR (100 MHz, D$_2$O)



IR

	^{13}C atom %
natural	1.109
excess	1.195

Fig. 3. The 100 MHz ^{13}C-NMR Spectrum of ^{13}C-Sodium Carbonate in Exhaust Gas from the Culture

In the structural formulas, ● = ^{13}C-enriched carbons. Solvent = D$_2$O; number of scans = 1000; acquisition time = 0.45 s; size of data table = 16 k points; pulse width = 45°. Shifts are relative to TSP.

in two ways, firstly by stereospecific incorporation into the pyranose ring of HA (**1a**), and secondly as an acetyl group, presumably *via* pyruvate and [^{13}CH$_3$]acetyl CoA.[11] These results confirm that the biosynthetic pathway of HA (**1a**) is as illustrated in Fig. 2.

The ^{13}C-NMR spectrum of the exhaust gas is shown in Fig. 3. The sharp signal of 171.2 ppm was assigned to ^{13}C-carbonate. The ^{13}C-atom % in the exhaust gas

measured by the $^{13}CO_2$ analyzer was 0.086% greater than that in natural sodium carbonate (1.195% in the exhaust gas and 1.109% in natural sodium carbonate) in spite of great dilution by ^{12}C-carbon dioxide during aeration. These measurements indicated that [1-^{13}C]-D-glucose (**2**) had been converted to ^{13}C-carbon dioxide, showing that some of the [1-^{13}C]-D-glucose (**2**) had been consumed as an energy source for this bacterium.

Experimental

Instruments ^{13}C-NMR spectra were taken on a JEOL GSX-400 spectrometer (100 MHz) and a GE NMR OMEGA-500 spectrometer (125 MHz). Chemical shifts are given downfield from sodium [2,2,3,3,-$^{2}H_4$]-3-(trimethylsilyl)propionate (TSP) as an internal standard for ^{13}C-NMR. IR spectra were measured with a $^{13}CO_2$ analyzer (JASCO EX-130).

Culture and Isolation of HA *S. zooepidemicus #104* was preincubated in 4 ml of brain heart infusion (BHI) medium at 37 °C for 18 h.

Next, 2.5 g of peptone, 1.25 g of yeast extract, 0.125 g of L-glutamine, and 230 ml of ion-exchanged water were placed in a 500 ml jar fermenter, and a few drops of Adecanol (Asahi Denka) were added as an antifoaming agent. This medium and 10 ml of [1-^{13}C]-D-glucose (**2**) solution (containing 8 g of [1-^{13}C]-D-glucose (**2**) (99% atom ^{13}C, Isotec, U.S.A.)) were each sterilized for 15 min at 121 °C.

The [1-^{13}C]-D-glucose (**2**) solution was added to the medium, and its container was washed with 10 ml of sterilized water. The washing was added to the jar fermenter, followed by 2 ml of the suspension of *S. zooepidemicus*. The culture was thoroughly stirred at 35 °C for 21 h. Aeration was accomplished by passing air (1 v/v/min (volume per volume per minute) at 20 °C) into the culture medium and the exhaust gas was passed through 150 ml of 2 N sodium hydroxide. The pH of the culture was kept constant at 7.0 by addition of 2 N sodium hydroxide from a pH stat.

When the pH change stopped, growth was stopped by adding 13.5 ml of benzyl alcohol. The medium volume was 265 ml, and 40 ml of 2 N sodium hydroxide had been consumed for pH control. The amount of 2 N sodium hydroxide used in the trap for exhaust gas was 148 ml. Next, active carbon (13.3 g) was added to the medium and the whole was stirred for 1 h. Methanol (265 ml) was added to the above mixture with stirring, then the cells and active carbon were removed by filtration. Sodium acetate trihydrate (44.1 g) was dissolved in the filtrate and 662.5 ml of methanol was added with stirring to give HA (**1a**) as a white precipitate. HA (**1a**) was collected by centrifugation (24000 × ***g***, 5 min), followed by lyophilization (yield, 1.888 g).

For ^{13}C-NMR spectroscopy, 12.0 mg of HA (**1a**) was washed with methanol and acetone and dissolved in about 0.5 ml of deuterium oxide (Fig. 1). ^{13}C-NMR (recorded at 125 MHz, in D_2O, internal standard TSP) δ: 23.2 (br s, N–CO$^{13}CH_3$ units), 101.3 (br s, [1-^{13}C]glucuronic acid units), 104.0 (br s, [1-^{13}C]glucosamide units).

The sodium hydroxide solution used to trap carbon dioxide was evaporated followed by freeze-drying to obtain 12.6 g of sodium carbonate. For ^{13}C-NMR spectroscopy, the sodium carbonate was dissolved to saturation in deuterium oxide (Fig. 3). ^{13}C-NMR (recorded at 100 MHz, in D_2O, internal standard TSP) δ: 171.2 (s, carbonate enriched peak).

Acknowledgements We thank Dr. M. Oyabu for ^{13}C-NMR measurement (125 MHz). We are also grateful to Mr. Y. Wachi, Mr. M. Yanagi, and Dr. S. Fukushima for suggestions concerning the culture.

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Protein Kinase Inhibitor H-7 Increases Lipoprotein Lipase Activity in Isolated Rat Fat Pads

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A protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) increased lipoprotein lipase (LPL) activity in isolated rat fat pads in a time- and dose-dependent manner. The incubation of H-7 with partially purified LPL did not affect its activity. Under the marked inhibition of protein synthesis by cycloheximide, H-7 still showed a full effect on the increase in LPL activity. A slight but significant increase in LPL activity in the fat pads was observed with inhibitors of cyclic nucleotide-dependent protein kinase. H-7, therefore, may increase LPL activity through processes other than the direct activation of the LPL molecule, or the stimulation of LPL molecule synthesis; probably through a decrease in the activity of protein kinases, especially protein kinase C.

Keywords H-7; protein kinase inhibitor; protein kinase C; lipoprotein lipase; adipose tissue

Introduction

Lipoprotein lipase (LPL, EC 3.1.1.34), which is localized on the membrane surface or the vascular endothelium in extrahepatic tissues, plays an important role in the metabolism of triacylglycerol-rich lipoproteins, including chylomicron and very low density lipoprotein (VLDL).[1-4] 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) is known to be a most potent inhibitor of protein kinase C (PKC) involved in the transduction of biological signals.[5] H-7 has been used to determine whether or not PKC is involved in various cellular responses in many experiments. Recently it has been reported that H-7 causes a depletion of normal active microfilament bundles in 3T3 cells,[6] and that H-7 potentiates the release of unsaturated fatty acids, such as oleic, linoleic and arachidonic acids, in A23187-stimulated neutrophils.[7] These reports show that H-7 appears to have effects in addition to inhibiting the response of PKC. It remains unclear whether the regulation of protein synthesis is involved in a part of the action of H-7. There are only a few reports on the relationship between LPL activity and PKC activity.

In this report, we show that H-7 stimulates LPL activity in rat fat pads without the elevation of protein synthesis.

Materials and Methods

Meterials L-[3,4,5,-^{3}H(N)]Leucine (5.31 TBq/mmol) was from New England Nuclear (Boston, MA). Cycloheximide was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Protein kinase inhibitors (H-7, H-8, and HA1004), which are derivatives of isoquinolinesulfonamide[3] were purchased from Seikagaku Industries, Ltd. (Tokyo, Japan). Staurosporine[8] was a gift from Dr. Matsuda (Kyowa Hakko Kogyo Co., Ltd., Tokyo). All other chemicals were of analytical grade.

Animals Male Wistar rats (200—220 g) were fed on a commercial laboratory chew *ad libitum* for a week and starved for 24 h before each experiment.

Preparation of Fat Pads and Enzyme Assay As described previously,[9] the fat pads, prepared from epididymal adipose tissue, were incubated with various agents at 37 °C for indicated periods in 2 ml of Kerbs–Ringer bicarbonate buffer containing 5 mM glucose and 2% bovine serum albumin. After incubation, the fat pads were homogenized on 30 mM Tris–HCl buffer, pH 8.5, 4 °C and the resultant supernatant was used as the enzyme preparation of LPL[9] and assayed using the colorimetric method.[10,11]

Results and Discussion

A linear increase in LPL activity was observed with the incubation of the fat pads with H-7 at a concentration of 100 μM for up to 180 min (Fig. 1). When the fat pads were incubated for 120 min with H-7 over a concentration range of 0—200 μM, LPL activity in the fat pads increased in a dose-dependent manner up to 200 μM (Fig. 2). To determine whether the direct action of H-7 on the LPL molecule is involved in the increase in LPL activity, the enzyme fraction was obtained from homogenized fat pads, as described in Materials and Methods, or prepared to partial purification by affinity chromatography on a heparin–Sepharose column.[12] The LPL activity of both fractions was never elevated by the addition of H-7 (data not shown). Fig-

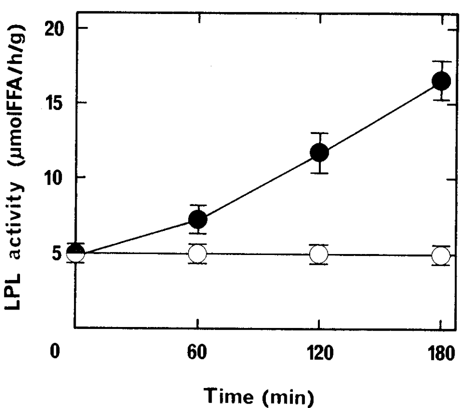

Fig. 1. Time Course of Increase in LPL Activity in the Fat Pads by H-7

The fat pads were incubated with H-7 (100 μM, ●) or without (○) for 0—180 min. The LPL activity in the fat pads in each case was determined.

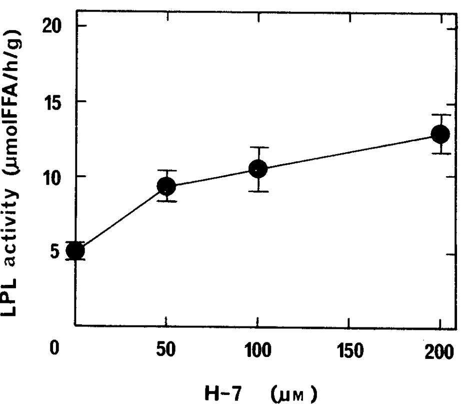

Fig. 2. Dose–Response Curve of the Increase in LPL Activity in Fat Pads by H-7

After the fat pads were incubated for 120 min at the indicated concentration of H-7, LPL activity in the fat pads was determined.

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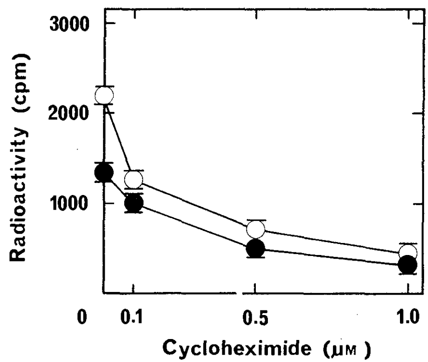

Fig. 3. Effect of Cycloheximide on Incorporation of [^{3}H]Leucine into the Protein of the Fat Pads Incubated with or without H-7

The fat pads were incubated with H-7 (100 μM, ●) or without (○) for 120 min at the indicated concentrations of cycloheximide under the addition of [^{3}H]leucine (18.5 kBq).[9,13] The incorporation of radioactivity into the protein in the fat pads was determined.

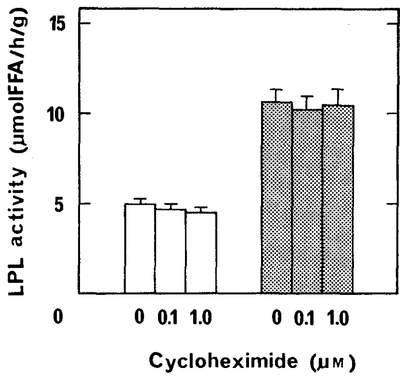

Fig. 4. Effect of Cycloheximide on H-7 Induced Increases in LPL Activity in Fat Pads

The fat pads were incubated with H-7 (100 μM, hatched bar) or without (white bar) for 120 min at the indicated concentrations of cycloheximide. LPL activity in the fat pads was determined.

ure 3 shows the inhibitory profiles by cycloheximide of [^{3}H]leucine into the protein of the fat pads. In the H-7-treated fat pads, the incorporation of [^{3}H]leucine into protein was reduced to 62% of the control by H-7, and was further decreased with an increase in the concentration of cycloheximide, similarly to non-treated fat pads. Although the protein synthesis was inhibited to 59 and 20% of the original level by 0.1 and 1 μM cycloheximide, respectively, the increase in LPL activity by H-7 was never inhibited (Fig. 4). These results show that the H-7-stimulated increase in LPL activity is dissociated from the stimulation of protein synthesis. The increase in LPL activity in fat pads by various protein kinase inhibitors is shown in Fig. 5. LPL activity in the fat pads was increased 1.5-fold by staurosporine, a PKC inhibitor different from isoquinolinesulfonamides.[8] A

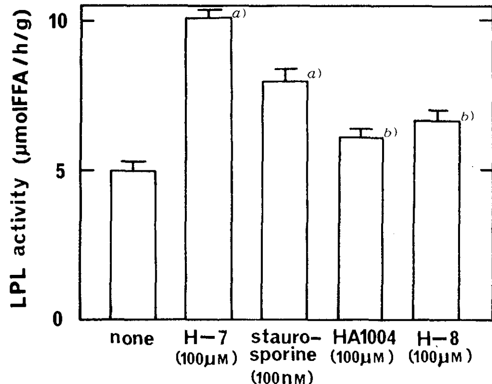

Fig. 5. Effect of Various Protein Kinase Inhibitors on LPL Activity in the Fat Pads

The fat pads were incubated with a protein kinase inhibitor, that is, H-8 (100 μM), H-7 (100 μM), HA1004 (100 μM), staurosporine (100 nM) or without. LPL activity in the fat pads was determined. Significantly different from no inhibitor at $p<0.01$ (*a*) and $p<0.05$ (*b*).

slight but significant increase in LPL activity was also observed with HA1004 and H-8, inhibitors of cyclic nucleotide-dipendent protein kinases.[3]

In conclusion, H-7 may increase the LPL activity through processes which involve neither direct activation of the LPL molecule, nor stimulation of LPL molecule synthesis; probably, the phosphorylation–dephosphorylation system including PKC is involved in the regulation of LPL activity.

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Occurrence of L-2,4-Diaminobutyrate Decarboxylase Activity in *Acinetobacter*

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Three strains of the genus *Acinetobacter* grown in a polyamine-free synthetic medium contained very high amounts of 1,3-diaminopropane, and there were also high concentrations in the extracellular growth medium. Little, if any, of the usual polyamines, putrescine, spermidine and spermine were found. There was no detectable activity of aminopropyltransferase (<0.2 nmol spermidine formed/mg protein/h), which would be responsible for the formation of spermidine or spermine, expected precursors of 1,3-diaminopropane. These observations suggested the possibility of another mode of 1,3-diaminopropane biogenesis. Decarboxylation activity towards L-2,4-diaminobutyrate leading to the formation of 1,3-diaminopropane was detected in extracts of all three strains examined. The decarboxylase was partially purified from *A. calcoaceticus* ATCC 23055. The enzyme was active against only L-2,4-diaminobutyrate among the diamino acids tested and required pyridoxal phosphate as a cofactor. Mg^{2+} activated the enzyme.

Keywords decarboxylase; *Acinetobacter*; 2,4-diaminobutyrate; 1,3-diaminopropane; polyamine

1,3-Diaminopropane (DAP) is one of the diamines present in nature and has been shown to be formed from either spermidine or spermine by the action of a dehydrogenase[1] or an oxidase.[2] However, we have previously reported that an enzyme which catalyzes the decarboxylation of L-2,4-diaminobutyrate (DABA) to yield DAP is present in *Vibrio* species[3] and *Enterobacter aerogenes*[4] which lack the ability to oxidatively cleave spermidine to DAP and Δ^1-pyrroline.

A strain of *Acinetobacter calcoaceticus* has been analyzed for its polyamines by Busse and Auling,[5] who detected a new polyamine pattern with DAP as a major component, and low contents of putrescine, spermidine and spermine. This unusual polyamine composition stimulated us to investigate the possible biosynthetic pathway for DAP in this genus. In this paper we present evidence that *Acinetobacter* possesses relatively high activity of DABA decarboxylase and this probably produces the large amounts of DAP. Measurements of the polyamine contents and aminopropyltransferase activity (spermidine synthase) of *Acinetobacter* supported the idea that DAP is not formed from spermidine or spermine.

Experimental

Chemicals Decarboxylated *S*-adenosylmethionine was kindly supplied by Prof. K. Samejima, Josai University, Saitama, Japan. All other chemicals were obtained as previously described.[4]

Bacterial Strains and Culture Conditions *A. calcoaceticus* ATCC 23055 and IFO 12552 and *A. lwoffii* ATCC 15309 were used. The preculture grown overnight in nutrient broth (Nissui, Tokyo, Japan) was added as an inoculum (2%, v/v) to the same medium to obtain cells for enzymatic studies. For polyamine analysis, a polyamine-free synthetic medium[6] containing 0.5% NaCl was used for both preculture and cell cultivation. Bacteria were grown by vigorous shaking at 30 °C for the times indicated. The foam formed during growth was destroyed with a few drops of octylalcohol and then the bacteria were harvested by centrifugation at $5300 \times g$ for 20 min at 4 °C.

Determination of Polyamines Polyamines in both cells and the culture supernatants were determined by a gas chromatographic (GC) method.[4]

Enzyme Assays Cells were sonicated at 4 °C in 5 volumes of 20 mM Tris–HCl (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 40 μM pyridoxal phosphate (buffer A). The homogenate was centrifuged at $40000 \times g$ for 30 min, and the supernatant was dialyzed for 12 h at 4 °C against 200 volumes of buffer A with two changes. This dialyzed extract was used for assays of the activities of both DABA decarboxylase and aminopropyltransferase. For DABA decarboxylase, the reaction mixture (1 ml) contained 100 mM Tris–HCl (pH 8.5), 0.2 mM pyridoxal phosphate, 10 mM $MgSO_4$, 15 mM DABA and enzyme protein (0.05—0.8 mg). The reaction was run at 37 °C for 30 min and was terminated by adding 0.4 ml of 20% $HClO_4$ and 1 ml of 1,6-diaminohexane (125 nmol/ml) as an internal standard. DAP in the supernatant was determined by a GC method.[4] Under the standard conditions, DABA decarboxylase activity was a linear function of the amount of protein or incubation time (*ca.* 60 min).

The aminopropyltransferase activity was assayed under the conditions of Tabor and Tabor.[7] The reaction mixture (0.5 ml), containing 50 mM Tris–HCl (pH 8.0—8.9), 0.5 mM putrescine, 0.2 mM decarboxylated *S*-adenosylmethionine and enzyme protein (*ca.* 2 mg), was incubated at 37 °C for 30 min and treated as above. Possible spermidine formation was determined by the GC method as previously described.[8]

Spermidine dehydrogenase and oxidase were assayed according to the methods of Okada *et al.*[9] and Smith,[10] respectively. The cell extracts were prepared under the same conditions as those of Okada *et al.*[9]

Partial Purification of DABA Decarboxylase All steps were carried out at 0—4 °C. Extract (118 ml) was prepared from *A. calcoaceticus* ATCC 23055 (24 g wet wt.) as described above. The precipitate formed between 45 and 70% $(NH_4)_2SO_4$ saturation was dissolved in about 10 ml of buffer A and then dialyzed overnight against 200 volumes of the same buffer with two changes. The dialyzed sample was applied to a DEAE-Sepharose CL-6B column (2.6 × 23 cm) pre-equilibrated with buffer A and eluted with a 500-ml linear gradient of 0—0.35 M NaCl in buffer A at a flow rate of 20 ml/min. To the combined active fraction was added $(NH_4)_2SO_4$ to 75% saturation, and the precipitate was dissolved in 1 ml of buffer A and dialyzed overnight against 100 volume of the same buffer with two changes. The dialyzed sample was applied to a Sephacryl S-300 column (2.1 × 80 cm) pre-equilibrated with buffer A containing 0.4 M NaCl and 0.02% NaN_3 and eluted with the same buffer at a flow rate of 10 ml/min. The combined active fraction (6 ml) was used for examination of some properties of the enzyme without removal of NaCl since the activity was not affected by its presence.

Other Methods The protein content of extracts was determined by the Lowry method and that of whole cells by its modification[11] with bovine serum albumin as the standard. Gas chromatography–mass spectrometry (GC-MS) analyses were done as previously described.[4]

Results and Discussion

Intra- and Extracellular Polyamine Contents Our results (Table I) agree with those of Busse and Auling,[5] who concluded that DAP is unusually abundant in *A. calcoaceticus* DSM 30006. However, unlike their results, we did not find measurable amounts of either spermidine or spermine in the cells (<0.2 nmol/mg cell protein). None of these polyamines was detected in the culture supernatants. Differences in the strain and/or the culture conditions could be responsible for the different results. We also found large amounts of DAP in the culture supernatants of these three strains (Table I). However, it is at present unclear whether DAP in the supernatants was actively excreted by the

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bacteria, lost by passive processes or attributable to cell lysis. Putrescine, a normal precursor of spermidine, was present in only small amounts (0.2—0.3 nmol/mg cell protein). In addition, the polyamine composition of each strain was unchanged at different stages of culture. In spite of high levels of intracellular DAP, norspermidine, which is suspected to be synthesized from DAP,[8,12] was undetectable in all the strains of *Acinetobacter* under any of the growth conditions studied.

Lack of Activity of Aminopropyltransferase and Spermidine Dehydrogenase and Oxidase The aminopropyltransferase activity was not detected in any phase of growth by the present assay method (detection limit: 0.2 nmol spermidine formed/mg protein/h). Extracts from *Escherichia coli* B used as a positive control revealed normal enzyme activity (52—67 nmol spermidine formed/mg protein/h).[7] Changes in the pH of the buffers used for sonication and enzyme assay did not reveal any activity. In agreement with these results, there was no appreciable increase in DAP or spermidine production, even when a large intracellular accumulation of putrescine (about 0.1 μmol/mg cell protein) was caused by the addition of putrescine (1 mM) to the medium. Moreover, no conversion of spermidine into DAP could be detected in cell extracts which were analyzed for spermidine dehydrogenase[9] and oxidase[10] activities. These observations strongly suggested the possibility of another mode of DAP biogenesis.

Decarboxylation Activity towards DABA When the strains were grown in the presence of added DABA, the DAP production increased markedly, a majority of that produced being detected in culture supernatants (Table I). This suggests that the organisms can convert DABA into DAP. In addition, the pyridoxal phosphate-dependent decarboxylation activity towards DABA leading to the formation of DAP was detected in dialyzed cell extracts of these strains (*ca.* 160—290 nmol/mg protein/h). The product appearing in the reaction mixture comigrated with authentic DAP and was identified as DAP by GC-MS. When the organisms were grown in the synthetic medium, a rapid increase in specific activity of this enzyme, termed DABA decarboxylase, closely corresponded to the onset of DAP accumulation seen 3 h after inoculation.

Table I. DAP Content in *Acinetobacter* and Enhanced Production of DAP by Addition of DABA to the Growth Medium

Strain[a]	Addition	DAP content (μmol/mg cell protein)	
		Cells	Supernatant
A. calcoaceticus ATCC 23055	None	0.208	0.229
	DABA (1 mM)	0.224	0.359
	DABA (5 mM)	0.246	1.921
A. calcoaceticus IFO 12552	None	0.213	0.789
	DABA (5 mM)	0.256	1.900
A. lwoffii ATCC 15309[b]	None	0.123	0.500
	DABA (5 mM)	0.155	0.632

a) The organisms were grown for 8 h at 30 °C in 200 ml of the polyamine-free synthetic medium. After harvesting the cells by centrifugation, DAP in both cells and culture supernatants was determined. *b*) Under the conditions used, the growth was relatively poor.

Partial Purification and Some Properties of DABA Decarboxylase DABA decarboxylase was partially purified from *A. calcoaceticus* ATCC 23055 according to the scheme shown in Table II. The enzyme purified in this way had an optimal pH in the range of 8.5—8.75 in 100 mM Tris–HCl. Lineweaver–Burk plots gave a K_m of 3.9 mM for L-DABA. The enzyme was assumed to be inactive on D-antiport of DABA (commercially unavailable), since the K_m for the racemate was more than 2-fold higher than that for L-DABA without an appreciable change in V. Neither L-2,3-diaminopropionic acid, L-ornithine nor L-lysine could serve as substrate at 15 mM, as judged by the observation that the possible reaction products, ethylenediamine, putrescine and cadaverine, were not detected by the GC method used ($<$0.5% of the reactivity for L-DABA). The enzyme showed no activity in the absence of pyridoxal phosphate. Near-maximal stimulation was obtained at concentrations above 0.1 mM pyridoxal phosphate and the K_m value was 9.1 μM. The enzyme was inhibited 42% and 78% by 0.5 and 1 mM carboxymethoxylamine, respectively. Pyridoxal phosphate was also essential for stabilizing the enzyme, but dithiothreitol had no effect. The enzyme was activated by Mg^{2+}, about 4-fold activation being observed at 5—10 mM.

It has been generally accepted that the oxidative cleavage of spermidine or spermine is the only known pathway for the production of DAP.[1,2] However, in addition to members of the genus *Vibrio*[3] and *E. aerogenes,*[4] three strains of *Acinetobacter* in this study have been proved to have DABA decarboxylase which participates in the formation of DAP. Circumstantial evidence also supported that in *Acinetobacter* spermidine (or spermine) is not a precursor of DAP. We also recognized this enzyme activity in other strains of *Enterobacter* such as *E. cloaceae* and *E. agglomerans*. These results suggest that DABA decarboxylase may be widely distributed in the bacterial kingdom to provide DAP. Like that of *E. aerogenes*, the enzyme partially purified from *A. calcoaceticus* was activated by Mg^{2+}. Preliminary observations have indicated that the native enzyme of *A. calcoaceticus* has a similar molecular mass to that of *E. aerogenes*. Studies are currently in progress to further characterize these enzymes. Although

Table II. Partial Purification of DABA Decarboxylase from *A. calcoaceticus* ATCC 23055[a]

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
Crude extract	679.4	182.8	0.27	1	100
$(NH_4)_2SO_4$ fractionation	129.6	179.5	1.39	5.1	98
DEAE-Sepharose CL-6B	14.0	145.4	10.4	38.5	80
Sephacryl S-300	2.44	110.8	45.4	168	61

a) A 24 g (wet wt.) portion of cells grown for 8 h was used as the starting material. Enzyme activity is expressed in units of μmol/h.

definitive identification of DABA in *Acinetobacter* has been unsuccessful to date and its biosynthetic pathway still remains to be determined, the close correspondence between the time of appearance of this enzyme activity and the accumulation of DAP strongly suggests a functional interrelationship. Our interest will be focused on the biosynthetic pathway for DABA as well as the physiological significance of such an unusual production of DAP in *Acinetobacter*.

Acknowledgements This work was supported in part by a General Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We are grateful to Mr. A. Iwadoh for his assistance in GC-MS analyses.

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Phospholipid Dependency of Hepatic Uridine Diphosphate-Glucuronyltransferase in the Developing Fetus of the Rat

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The developmental change of uridine diphosphate-glucuronyltransferase (UDPGT) was studied using hepatic microsomes of rat fetuses on days 18 and 21 of gestation. Total phospholipid content was higher on day 21 than on day 18, although no significant difference in the composition between the two stages was observed. Lipid removal with phospholipase A_2 greatly reduced UDPGT activity for phenol in the later stage of gestation, even though the delipidation ratios in both stages were similar. The fluidity in the hydrocarbon region of the microsomal membrane was higher on day 21 than on day 18. No significant difference in the polarization of *N*-dansylaziridinyl protein was observed between the stages.

Keywords uridine diphosphate-glucuronyltransferase; rat fetus; delipidation; fluidity; phospholipid; hepatic microsome; protein polarization

It is well known that uridine diphosphate-glucuronyltransferase (UDPGT) located in the microsomal membrane is phospholipid dependent.[1] The developmental change in phospholipid dependency of this enzyme in the perinatal period of the rat was studied using detergent.[2] This work investigated the developmental change in the phospholipid dependency of UDPGT of rat fetuses on days 18 and 21 of gestation using phospholipase A_2. A fluorescence polarization method has been applied to the study of microsomal membranes in the perinatal period.[3] This work attempted to examine the fluidity in various regions of the microsomal membrane.

Experimental

Animals Pregnant Wistar rats on days 18 and 21 of gestation were used.

Materials Phenol-^{14}C-(U) (specific activity, 100 mCi/mol) was purchased from NEN Research Products. Phospholipase A_2 and *N*-dansylaziridine were purchased from Sigma. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich. 2-(9-Anthroyloxy)stearic acid (2AS) and 12-(9-anthroyloxy)stearic acid (12AS) were purchased from Wako (Japan).

Preparation of Microsomes Fetal liver was homogenized in 3 volumes of 0.15 M KCl/0.05 M Tris HCl (pH 7.5), and a microsomal fraction was prepared by centrifugation in the manner described previously.[4]

UDPGT Activity The enzyme activity for ^{14}C-phenol (specific activity, 1.6 mCi/mmol) was measured in the manner described previously.[4] The reaction was stopped by the addition of a half volume of methanol and then centrifuged at 3000 rpm. The supernatant was subjected to thin layer chromatography (TLC).

TLC and Radioactivity Assay Plates (Merck Silica gel F_{254}) were developed in *n*-butanol–15% ammonia water (5 : 1). The radioactivity of the glucuronide was assayed in the manner described previously.[4]

Phospholipase A_2 Digestion Delipidation of the microsome with phospholipase A_2 was carried out as described by Erickson *et al.*[5]

Phospholipid Analysis Microsomal lipids were extracted into chloroform–methanol (2 : 1).[6] Total phospholipid contents were determined by the method of Bartlett.[7] Phosholipids were separated according to Graham *et al.*[8]

Fluorescence Measurement Microsomes (400 mg of protein) were incubated with DPH (6 μmol) in 4 ml of 10 mM Tris HCl (pH 7.4) containing 1 mM ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid at 20 °C for 30 min. For measurements of 2AS and 12AS, microsomes (770 mg of protein) were incubated with probes (10 nmol) in 4 ml of 10 mM Tris HCl (pH 7.4) at 20 °C for 20 min. A value of $(r/r_0-1)^{-1}$, fluidity parameter, was calculated by the methods of Charnock and Bashford,[9] and of Schachter and Shinitzky.[10] For fluorescent measurement of microsomal protein, thiol groups of protein (5 mg) were reacted with *N*-dansylaziridine (2.5 mg), and polarization was assayed according to the report of Scouten *et al.*[11]

Results and Discussion

UDPGT, a phospholipid dependent enzyme, is thought to be influenced by the developmental change in biochemical and physicochemical changes of the microsomal membrane during development of the fetus. Thus, phospholipid content in the hepatic microsomes of fetuses on days 18 and 21 of gestation was first measured. As shown in Table I, phospholipid content in the native microsome was higher on day 21 of gestation than on day 18. Delpech *et al.*[12] reported no significant change in phospholipid content from day 16 of gestation to day one after birth, whereas Kapitulnik *et al.*[3] reported that phospholipid content increased by 47% between day 21 of gestation and day one after birth. The present data are related to the results of Kapitulnik *et al.*, and indicate that the phospholipid synthesis in the fetal liver increases with the days of gestation.

The developmental change in phospholipid dependency of UDPGT was studied by perturbing phospholipids with detergent.[2] This author undertook to examine the influence of phospholipids on UDPGT activity by removing phospholipids with phospholipase A_2. When phospholipids

Table I. Effect of Delipidation on Phospholipid Contents in Hepatic Microsome of Developing Fetus of the Rat

Gestation day	Phospholipid content (P nmol/mg protein)		
	Native	Delipidated	Decreased ratio (%)
18	118±23	21.8± 5.0[a]	81.5
21	201±31	53.7±11.2[a]	73.3

Data are the mean±S.E. of 5 l. *a*) Significantly different from the value of the native microsome ($p<0.05$).

Table II. Effect of Delipidation on UDPGT Activity of Developing Fetus of the Rat

Gestation day	Activity (nmol/min·mg protein)		
	Native	Delipidated	Decreased ratio (%)
18	3.22±0.38	2.21±0.19[a]	31.4
21	16.68±2.73	3.05±0.49[a]	81.6

Data are the mean±S.E. of 5 l. *a*) Significantly different from the value of the native microsome ($p<0.05$).

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Table III. Composition of Hepatic Microsomal Phospholipids of Developing Fetus of the Rat

	Phospholipid composition (%)	
	18th day	21st day
Phosphatidylcholine	59.6	56.3
Phosphatidylethanolamine	14.2	16.5
Sphingomyelin	5.3	4.8
Lysophosphatidylcholine	4.7	4.4
Phosphatidylinositol + phosphatidylserine	16.2	14.0

Data are the percentage of total phospholipid P.

Table IV. Fluidity of Hepatic Microsomal Membrane of Developing Fetus of the Rat

Probe	$(r_0/r-1)^{-1}$	
	18th day	21st day
DPH	0.366 ± 0.010	$0.291 \pm 0.009^{a)}$
2AS	0.951 ± 0.014	0.939 ± 0.012
12AS	0.825 ± 0.012	$0.743 \pm 0.010^{a)}$

Data are mean ± S.E. of five experiments. *a*) Significantly different from the value of the 18th day of gestation ($p < 0.05$).

in the microsome were digested with phospholipase A_2, phospholipid content decreased by 81.5% of the native microsome by day 18 of gestation, and by 73.3% by day 21, showing no marked difference in the ratio of decrease between the two stages.

Table II shows UDPGT activities for phenol in native and delipidated microsomes on the two gestational stages. UDPGT activity on the native microsome on day 21 was higher than that on day 18, in agreement with the results using other substrates.[13] UDPGT activities on both stages of gestation were decreased by delipidation; the effect of delipidation was higher on day 21 than on day 18, although no significant difference in the removal ratio of phospholipids between the stages was observed (Table I). This result suggests that UDPGT on the later stage of gestation interacts more tightly with phospholipids than that on the earlier stage.

It is noteworthy that fluidity is dependent on the species of phospholipid.[14] Thus, the composition of microsomal phospholipids was measured to determine if it changed during development of the fetus. Table III shows the phospholipid composition in the native microsomes on both stages of gestation; no marked difference was apparent. This result would relate to that of Kapitulnik *et al.*[3] that there was no significant difference in phospholipid composition between the fetus on day 21 of gestation and a newborn within 24 h after birth. Thus, the composition of microsomal phospholipids presumably has no effect on the developmental change of the phospholipid–UDPGT interaction.

The relationship between membrane-bound enzymes and membrane fluidity was studied using DPH.[3] In addition to DPH, this work used 2AS, reflecting the fluidity in the hydrocarbon region close to the polar interface of the membrane, and 12AS, reflecting the fluidity in the region distant from polar head.[15] Table IV shows fluidity parameters of fluorescent probes. The parameter of DPH on day 21 of gestation was lower than that on day 18, supporting the result of Kapitulnik *et al.*[3] that fluidity changed between the 19th and the 21st day of gestation. The parameter of 12AS on day 21 was lower than that on day 18, whereas the parameter of 2AS showed no significant difference between the two stages. This difference suggests that the hydrocarbon region distant from the polar/nonpolar interface of the membrane increased in fluidity in the later stage of gestation.

To examine the influence of phopholipid on the flexibility of microsomal protein, fluorescence polarization of thiols of protein modified with *N*-dansylaziridine was measured. The value of polarization was 0.372 ± 0.014 on day 21 of gestation and 0.359 ± 0.010 on day 18, showing no significant difference between the two stages. Thus, the difference in the phospholipid/protein ratio between the two gestational stages has no significant influence on the degree of rigidity of the protein molecule.

The relationship between phospholipids and UDPGT activity has been discussed from viewpoints of compartmentation and phospholipid constraint.[16] From the present data, it is reasonable to suggest that the increase of phospholipid/microsomal protein ratio with the day of gestation is associated with the formation of microenvironmental conformation of UDPGT with high activity.

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 Chem. Pharm. Bull. 39(9) 2456—2458 (1991) Vol. 39, No. 9

The Transport of a Drug to the Cerebrospinal Fluid Directly from the Nasal Cavity: The Relation to the Lipophilicity of the Drug

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The objective of the present study was to clarify the relation between drug transport to the cerebrospinal fluid (*CSF*) from the nasal cavity and the lipophilicity of the drug using hydrophilic sulfonamides as model durgs. The nasal cavity of the rat was perfused in a single pass system and the concentrations of sulfonamides in plasma and *CSF* were measured. The drug concentrations in *CSF* and plasma after nasal perfusion were compared with those after intravenous (i.v.) administration. The drug concentrations in the *CSF* were remarkably high after nasal perfusion in comparison with those after i.v. administration, though the time course of the plasma concentration was not much different from that after i.v. administration. These results suggested the existence of a direct transport pathway of the sulfonamides from the nose to the *CSF*. In addition, the drug concentrations in the *CSF* increased with increasing the lipophilicity of the drugs (the partition coefficient (*Pc*) of the drugs between isoamyl alcohol and pH 7.4 phosphate buffer). A significant correlation was observed between the drug concentrations in *CSF* and *Pc*. In conclusion, the direct transport pathway of the sulfonamides from the nose to the *CSF* was confirmed and, with regard to drugs with comparatively low lipophilicity, the degree of the transport depended on its *Pc*.

Keywords nasal administration; nasal absorption; nasal cavity; cerebrospinal fluid; drug delivery; cisternal puncture; sulfonamide; lipophilicity; passive diffusion

Introduction

There exists much evidence showing the presence of a drug transport pathway from the nose to the cerebrospinal fluid (*CSF*). Kumar *et al.* have demonstrated that progesterone and estradiol achieve higher levels in the *CSF* following intranasal administration compared to intravenous (i.v.) administration.[1,2] They also showed that the concentration of dopamine in *CSF* after spraying into the nostril of rhesus monkeys was remarkably high in comparison with the concentration after i.v. administration.[3] Some physiological data shows that the cerebral perivascular space and subarachnoid space of the olfactory lobes are connected with the submucous bases of the nose.[4,5] On the basis of these reports, Pardridge suggested in his review that intranasal administration was a possible route for the delivery of a drug to the brain.[6,7] However, details of drug transport from the nasal cavity to *CSF* has been unknown. In the previous paper, we confirmed the presence of a direct transport pathway from the nasal cavity to the *CSF* using cephalexin as a model drug.[8] In this report, the transport of sulfonamides with various degrees of lipophilicity was investigated with *in situ* nasal perfusion.

Materials and Methods

Chemicals Sulfisoxazole (SIX), sulfamethizole (SMZ) and sulfisomidine (SID) were purchased from Sigma Chemical Company. Sulfanilic acid (SA) was obtained from Wako Pure Chemical Industries, Ltd. All the other chemicals were of commercially available analytical grade.

Animal Preparation Male Wistar rats weighing 220—260 g were used. The rat was anesthetized with intraperitoneal pentobarbital (50 mg/kg) and the right femoral artery was canulated with polyethylene tubing (SP-31).

Nasal Perfusion Experiment The surgical operation was carried out on the esophagus and trachea as described by Hirai *et al.*[9] The nasal cavity was perfused with an isotonic drug solution (pH 7.4 phosphate buffer, drug concentration 10 mM) at a flow rate of 1 ml/min in a single pass system. Blood was taken from the femoral artery periodically (15, 30, 45, 60 min). Sixty minutes after starting the perfusion, *CSF* was taken by cisternal puncture as previously reported.[8]

i.v. Administration Experiment Sulfonamides (SA, SMZ, SIX 1 mg/rat; SID 1.5 mg/rat) were administered intravenously *via* the left femoral vein, and plasma was collected periodically. Sixty minutes after administration, *CSF* was obtained as described above.

Partition Coefficient (*Pc*) The *Pc* was determined as previously reported using pH 7.4 phosphate buffer and isoamyl alcohol as water and organic phases, respectively.[10]

Analytical Methods Sulfonamides in *CSF* and plasma were diazotized and coupled with *N*-1-naphthyl-*N'*-diethylenediamine (Tsuda reagent) according to a standard procedure[11] with some modifications.

***CSF* and Plasma** The blood was centrifuged for 5 min and its plasma was mixed with an equal volume of 10% trichloroacetic acid for deprotenization. The mixture was recentrifuged to obtain the supernatant. Twenty microliters of 0.7 N HCl was added to 70 μl of *CSF* or a deprotenized plasma sample and then cooled to 0 °C. Ten microliters of 0.2% sodium nitrite, 20 μl of 0.2% ammonium sulfate and 10 μl of 0.2% Tsuda reagent were added and mixed well at 3 min intervals. The diazo reactants were kept at room temperature for 30 min. Twenty microliters of the reactant solution, injected with the sample injector (model 7125, Rheodyne, CA, U.S.A.), was passed into the flow cell of a spectrophotometric detector (SPD-6AV, Shimadzu) by a pump (LC-6A, Shimadzu) at a flow rate of 1.5 ml/min. The optical density of the reactant solution was recorded and analyzed by a Chromatopack (C-R2AX, Shimadzu) to obtain the concentration of the sulfonamides.

TABLE I. Chemical Structure and Partition Coefficient of Sulfonamides

		Pc
Sulfanilic acid (SA)	$H_2N-C_6H_4-SO_3H$	0.012
Sulfamethizole (SMZ)	$H_2N-C_6H_4-SO_2-NH-$(5-methyl-1,3,4-thiadiazol-2-yl)	0.250
Sulfisoxazole (SIX)	$H_2N-C_6H_4-SO_2-NH-$(dimethyl-substituted ring, CH_3, CH_3)	0.261
Sulfisomidine (SID)	$H_2N-C_6H_4-SO_2-NH-$(dimethylpyrimidinyl, CH_3, CH_3)	0.892

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TABLE II. The Time Courses of Plasma Concentrations, AUC and the Concentration in CSF 60 min after Starting the Nasal Perfusion

Drug	Plasma (μM)				AUC_{0-60} (μM·min)	CSF (μM)
	15 min	30 min	45 min	60 min		
SA	2.21±0.43	5.16±0.78	8.67±1.42	12.32±1.77	333.1± 54.7	3.01±0.51
SMZ	6.79±0.50	12.46±1.63	21.52±1.95	28.08±2.34	822.1± 76.1	5.50±0.68
SIX	26.60±1.79	52.79±3.33	74.98±5.18	96.91±6.79	3042.3±200.1	6.43±1.58
SID	42.74±3.15	73.31±4.30	93.07±3.03	106.69±4.04	3959.5±124.0	9.71±0.85

Data represents mean±S.E. of 4—6 rats. AUC_{0-60} was calculated based on the linear trapezoidal rule.

TABLE III. The Time Courses of Plasma Concentrations, AUC and the Concentration in CSF 60 min after Intravenous Administration

Drug	Plasma (μM)				AUC_{0-60} (μM·min)	CSF (μM)
	15 min	30 min	45 min	60 min		
SA	25.67±2.05	15.02±1.09	11.87±1.15	8.83±1.62	1117.2± 82.9	0.640±0.082
SMZ	37.03±2.23	27.85±3.21	25.54±4.48	24.41±2.94	1873.0±153.0	0.388±0.092
SIX	85.05±2.62	69.95±3.73	65.67±2.19	63.65±1.99	4456.0±164.6	0.407±0.037
SID	116.01±3.87	100.63±4.43	84.36±4.26	80.47±1.80	6040.6±228.9	0.645±0.046

Data represents mean±S.E. of at least 3 rats. The concentration at 0 min was estimated by extrapolation of the logarithms of the plasma concentration at 15, 30, 45, and 60 min, and AUC_{0-60} was calculated based on the linear trapezoidal rule.

Results

Comparison of the Concentration in the Plasma and *CSF* after Nasal Perfusion with the Concentration after i.v. Administration The drug concentrations in plasma and CSF after nasal perfusion and after i.v. administration are summarized in Tables II and III, respectively. The areas under the plasma concentration–time curve (AUC) are also shown in Tables II and III. The concentration in CSF after i.v. administration was very low, and no significant difference was observed among the sulfonamides. However, the concentrations in CSF after nasal perfusion were remarkably high compared with those after i.v. administration, though AUC_{0-60} were high and the time courses of the plasma concentrations were not much different.

The Relation between the Lipophilicity of the Drug and Its Transfer to *CSF* Figure 1 shows the relation of the drug concentrations in CSF to the Pc of the drugs. The drug concentrations in CSF increased as the Pc of the drugs increased. A significant correlation was observed between Pc and the drug concentrations in CSF ($p<0.05$ by a Student's t-test).

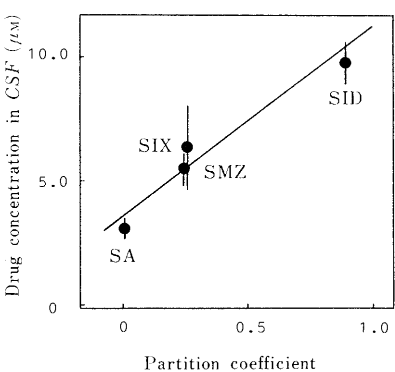

Fig. 1. The Relation of the Concentration in CSF to the Partition Coefficient for Sulfonamides

Each point represents the mean±S.E. of 5—7 rats. The line is the least-squares fit to data and the correlation coefficient is 0.9676.

Discussion

In the previous paper, we investigated the transport pathway of cephalexin from the nose to the CSF by comparing the concentrations in plasma and CSF after nasal, i.v. and intraduodenal administrations. The concentration of cephalexin in CSF 15 or 30 min after nasal administration was hundred-fold higher than the concentrations after i.v. and intraduodenal administrations, showing that there exists a direct transport pathway from the nose to the CSF and that the transport from the nose to the CSF is comparatively rapid. Kumar *et al.* showed that dopamine could be detected in the CSF within 15 min after nasal spraying.[3] Our finding is consistent with their results. In this paper, the existence of a direct transport pathway of the sulfonamides was also confirmed. Sulfonamides achieved remarkably higher levels in the CSF after nasal perfusion as compared to i.v. administration. It was also shown that the concentration in the CSF was the thousandth of that in the nasal perfusion fluid (10 mM). Furthermore, the degree of the drug transfer from the nose to the CSF was dependent on the Pc of the drugs. A significant correlation was observed between the drug concentration in the CSF and the Pc of the drug. However, the preliminary experiment showed that the concentration of a drug with a higher Pc in CSF after nasal perfusion was low in comparison with that expected from the relation shown in Fig. 1, showing the complexity of the drug transport from the nose to the CSF. This may be partly due to the rapid absorption of the drug by the systemic circulation.

In conclusion, there exists a direct transport pathway of sulfonamides from the nose to the CSF and, with regard to drugs with a comparatively low lipophilicity, the degree of transport was dependent on their Pc. In addition, the findings also provide information on the drug's side effects on the central nervous system when they are administered nasally.

Acknowledgment This work was supported by a Grant-in-Aid for

Scientific Research from the Ministry of Education, Science and Culture, Japan.

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TRIFLUOROMETHYLATION OF CHIRAL ALDEHYDE AND SYNTHESIS OF 6-DEOXY-6,6,6-TRIFLUOROHEXOSES

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Trifluoromethylation of chiral aldehyde derived from sugar was efficiently carried out by a modification of the reported procedure. The preparation of 6,6,6-trifluoro-L-daunosamine was also achieved by using (2*S*,3*R*)-4,4,4-trifluorobutane-1,2,3-triol derivative.

KEYWORDS trifluoromethylation; ultrasound; slow addition; 6-deoxy-6,6,6-trifluorohexose; 6,6,6-trifluoro-L-daunosamine

Sugar derivatives containing the trifluoromethyl group are considered to be attractive molecules not only for important building blocks in the preparation of optically active fluoro molecules but also for assessment of expected biological activity. Many kinds of the monofluoro- and difluorosugar analogues have been studied to assess the biological activity of sugar analogues and/or glycosyl compounds.[1)] The preparations of optically active trifluoromethylated carbinols have been developed extensively in recent years,[2)] but elaboration on producing enantiomerically pure trifluoromethylated carbinols in a simple and effective manner remains a task for the synthesis of 6-deoxy-6,6,6-trifluorohexose derivatives.[3)] We describe herein the trifluoromethylation of a chiral aldehyde and the synthesis of 6-deoxy-6,6,6-trifluorohexose derivatives involving 6,6,6-trifluoro-L-daunosamine.

Trifluoromethylation was carried out efficiently by introducing trifluoromethyl iodide slowly (0.5 ml/0.5 h) to a mixture of zinc and aldehyde in dimethylformamide (DMF) under the irradiation of ultrasound. This simple modification of the original procedure[4)] by the slow addition of trifluoromethyl iodide is indispensable, because none of the desirable products can be obtained by the original procedure. For example, the aldehyde **3**[5)] was trifluoromethylated in 47% yield to give a separable mixture of diastereoisomers (**4a** and **4b**) in a 1 : 2.5 ratio and the α,β-unsaturated aldehyde **5** (20%) (Chart 1). Trifluoromethylation of 2,3-*O*-cyclohexylidene-D–glyceraldehyde also gave a mixture of 2*R*,3*R*- and 2*R*,3*S*-isomers (**6a** and **6b**, 70 % yield, ratio 3 : 2).

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70% (3 : 2)

6a (2*R*, 3*R*) $[\alpha]_D^{23}$ 13.2° (c=0.65, $CHCl_3$)
6b (2*R*, 3*S*) $[\alpha]_D^{23}$ −14.8° (c=0.74, $CHCl_3$)

Chart 1

The absolute configuration of the newly formed chiral center of **4a** was determined to be the *S*-configuration by X-ray analysis of the corresponding p-nitrobenzoate of **4a**[6]. The structures of **6a** and **6b** were determined by converting these compounds to the methyl ether **7a, b** followed by comparison of the spectral data and rotational values ($[\alpha]_D$) with those of compound **7a** (2*S*, 3*S*) derived from **4a** (Chart 2). Under the same trifluoromethylation conditions, other aldehydes[7] derived from sugars also gave a mixture of diasetereoisomeric products, and the absolute configuration of each diastereoisomer was unambiguously determined in the same way as in Chart 2.

4a R=PMB $[\alpha]_D^{23}$ -31.6° (c=1.62, $CHCl_3$); 1) MeI/NaH 2) Ce^{IV} 3) H_3O^+ 4) $NaIO_4$; $LiAlH_4$ → 7a (2*S*, 3*S*) $[\alpha]_D^{23}$ -15.6° (c=0.54, $CHCl_3$)

6a,b: 1) MeI/NaH 2) H_3O^+ → 7a (2*R*, 3*R*) $[\alpha]_D^{23}$ 14.5° (c=0.38, $CHCl_3$); 7b (2*R*, 3*S*) $[\alpha]_D^{23}$ −7.2° (c=0.29, $CHCl_3$)

Chart 2

As one synthetic application of trifluoromethylated polyols, the preparation of 6,6,6-trifluoro-L-daunosamine (**8**),[8] which is the sugar moiety of anthracycline antibiotics, was carried out from **6b** (2*S*, 3*R*) obtained from 2,3-*O*-cyclohexylidene-L-glyceraldehyde (Chart 3). In the synthesis of **8**, the two points (oxidation of **9** and allylation of imine **10**) deserve comment. i) In the workup of the oxidation of primary alcohol **9** to aldehyde by Swern oxidation, termination of the reaction by adding the reaction mixture to ice-H_2O at 0°C to avoid the epimerization was necessary. ii) The allylation of **10** with allylmagnesium bromide yielded olefin (**11**) with low *syn*-selectivity (*syn*/*anti* = 3/2) due to α-chelation control.[9]

6b (2*S*, 3*R*) $[\alpha]_D^{23}$ +15.5 (c=2.97, $CHCl_3$) —a, 60%→ 9 $[\alpha]_D^{23}$ -7.0 (c=2.21, $CHCl_3$) —b, c→ 10 —d→ 11 79% (from 9), 4,5-*syn*/*anti*=1/4 —e, 80%→ 8 80% (from *anti*-isomer), β/α=6/1[8]

PMB=p-methoxybenzyl
PNBz=p-nitrobenzoyl

Chart 3. Reagents and Conditions: a; i PMBCl/NaH, ii H_3O^+ iii PivCl/Py, iv $CH_2(OMe)_2/P_2O_5$ v $LiAlH_4$ b; Swern Oxid. c; $BnNH_2$ d; allyl-$B(OiPr)_2$ e; i TFAA/ Et_3N/DMAP, ii DDQ/CH_2Cl_2–H_2O, iii O_3 iv Me_2S, v PNBzCl/Py

Although the biological assay of 6-deoxy-6,6,6-trifluorohexoses (**1a**, **1b**, **2a** and **2b**),[10] prepared by the present procedure, showed no significant inhibitory activity toward L1210 leukemia cells,[11] we believe that these 6-deoxy-6,6,6-trifluorohexoses are considered to play an important role in specifying the function of a sugar moiety such as the L-fucose involved in cell-surface oligosaccharides.

ACKNOWLEDGEMENT We wish to express our appreaciation to Dr. Akihiro Yoshimoto at Mercian Corporation for conducting the *in vitro* assay of the synthesized 6-deoxy-6,6,6-trifluorosugars.

ADDED IN PROOF (Aug. 14, 1991) Since submission of this manuscript, preparations of 6,6,6-trifluorohexoses which are the same analogues prepared by us has appeared. R. C. Bansal, B. Dean, S. Hakomori and T. Toyokuni, *J. Chem. Soc., Chem. Commun.*, **1991**, 796.

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6) X-ray crystal data of p-nitrobenzoate of **4a**: $C_{23}H_{22}NO_8F_3$, M=497.422, orthorhombic, $P2_12_12_1$, a=10.953(1) Å, b=27.711(2) Å, c=7.875(1) Å, V=2390.2 Å^3, Dc=1.382 g/cm^3, Z=4, Cu Kα (λ=1.54178 Å). The structure was solved by direct methods and refined by a block-diagonal least squares method to R=0.054 for 1702 observed reflections [Fo>3σ(Fo)].

7) Aldehydes (**i** , **ii**) were also trifluoromethylated in 51~55% yields.

8) Care should be taken in regard to the α–, β–definition for L–sugar derivatives. Spectral data of β-anomer of **8**: ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ring protons); 5.99 (1H, dd, J=2.2 and 9.8Hz, 1-H), 4.66 (1H, ddd, J=2.0, 3.7 and 13.7Hz, 3-H), 4.47 (1H, d, J=2.0, 4-H), 4.08 (1H, q, J_{H-F}=6.1Hz, 5-H), 2.41 (1H, ddd, J=3.7, 12.3 and 13.7Hz, 2-H_{ax}), 1.74 (1H, ddd, 2.2, 3.7 and 12.3Hz, 2-H_{eq}). ^{19}F-NMR ($CDCl_3$) ppm (higher field from external benzotrifluoride signal was expressed as negative); -6.1 (s) and -10.6 (d, J=6.1Hz), 1:1 ratio. $[\alpha]_D^{23}$ -14.5° (c=0.77, $CHCl_3$). α-Anomer of **8**; 6.51 (1H, dd, 1.5 and 2.3Hz, 1-H), 4.92 (1H, ddd, 2.5, 4.0 and 13.7Hz, 3-H), 4.55 (1H, d, J=2.5Hz, 4-H), 4.35 (1H, q, J_{H-F}=6.4Hz, 5-H), 2.54 (1H, ddd, J=2.3, 13.7 and 13.8Hz, 2-H_{ax}), 1.62 (1H, ddd, J=1.5, 4.0 and 13.8Hz, 2-H_{eq}). ^{19}F-NMR ($CDCl_3$) ppm; -6.1 (s) and -11.1 (d, J=6.4Hz), 1:1 ratio. $[\alpha]_D^{23}$ -158.1° (c=0.23, $CHCl_3$).

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10) 6-Deoxy-6,6,6-trifluoro-D-galactose **1a**, mp 125–126°C, $[\alpha]_D^{23}$ 35.2° (c=0.20, H_2O); 6-deoxy-6,6,6-trifluoro-L-altrose **1b**, oil $[\alpha]_D^{23}$-7.0° (c=0.40, H_2O), 6-deoxy-6,6,6-trifluoro-D-altrose **2a**, oil $[\alpha]_D^{23}$ 8.5° (c=0.30, H_2O), 6,6,6-trifluoro-L-fucose **2b**, mp 121–124°C, $[\alpha]_D^{23}$-33.0° (c=0.70, H_2O).

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(Received June 10, 1991)

RADICAL CYCLIZATIONS TO FLUOROOLEFINS

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Radical cyclizations were effectively applied to systems containing fluoroolefins (α-fluoro-α,β-unsaturated ester, fluoroallyl acetate, *gem*-difluoroolefin, and fluorine-substituted unsaturated lactone) for the synthesis of ring compounds containing fluorine-substituted groups.

KEYWORDS radical cyclization; fluoroolefin; fluorine-containing ring compound; high dilution method; bicyclolactone; Corey lactone

Radical cyclizations are of considerable interest due to their synthetic potential for ring formation.[1] Specific chemoselectivity under free-radical conditions renders them applicable to substrates containing various functionalities. The stability of the C-F bond under the conditions of the tin hydride method has made it possible to investigate the radical cyclization of fluorine-substituted systems.[2] Fluoroolefin derivatives, easily prepared by Wittig type reactions, are building blocks commonly used in organofluorine chemistry. However, their applications to radical cyclization have not been well documented.[2a] This paper describes radical cyclizations involving fluoroolefins [α-fluoro-α,β-unsaturated ester (**1**), fluoroallyl acetate (**2**), *gem*-difluoroolefin (**3**), and fluorine-substituted unsaturated lactone (**4**)] as a radical acceptor.

F, CO_2Et **1**; F, OAc **2**; F, F **3**; O, O, F **4**

The substrates (**7**-**9**) containing fluoroolefin moieties (**1**-**3**) were prepared by Wittig ($Ph_3P{=}CF_2$)[3] or Emmons reactions [$(EtO)_2P(O)CHFCO_2Et$ (**6**)][4] with aldehyde derivatives (**5**) and the subsequent transformation reactions of functional groups.

X, CHO **5** (X=OTHP or X=OAc) —[$(EtO)_2P(O)CHFCO_2Et$ (**6**) or $ClCF_2CO_2Na$, Ph_3P]→ X, F, Y (X=OTHP, Y=CO_2Et or X=OAc, Y=F) → I, F, Y: **7** (Y=CO_2Et), **8** (Y=CH_2OAc), **9** (Y=F)

Radical cyclization of the substrates (**7**, **8**, and **9**) was carried out by 1.2 eq of Bu_3SnH and a catalytic amount of AIBN in benzene at reflux temperature for 3 - 5 h.[5] Reactions of unsaturated ester derivatives (**7a**

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and **7b**) in a 0.02 M solution of Bu_3SnH gave the cyclopentane and cyclohexane derivatives (**10a**[6] and **10b**) via selective exo cyclization in 91% and 89% yields, respectively. Similarly, the allyl acetate derivative (**8a**) under the 0.02 M conditions gave a high yield (88%) of cyclopentane derivative (**11a**) via 5-exo cyclization.

7a (n=1), **7b** (n=2) → Bu_3SnH, cat. AIBN [0.02 M] → **10a** (91%), **10b** (89%)

8a (n=1), **8b** (n=2) → Bu_3SnH, cat. AIBN → **11**
[0.02 M] **11a** (88%)
[0.02 M] **11b** (32%), **12b** (57%)
[0.002M] **11b** (77%), **12b** (2%)
[$CH_3(CH_2)_3CH(Ph)CH{=}CFCH_2OAc$ (**12b**)]

9a (n=1), **9b** (n=2) → Bu_3SnH, cat. AIBN →
[0.04 M] **13a** (70%)
[0.04 M] **13b** (10%)
[0.003 M, slow add.] **13b** (43%)

On the other hand, 6-exo cyclization of **8b** afforded a 32% yield of **11b** under the same conditions (0.02 M) along with the reduction product (**12b**) in 57% yield. The high dilution method for the cyclization of **8b** significantly improved the yield of **11b** by suppressing the reduction of the initial carbon radical with Bu_3SnH.[7] Thus, lowering the concentration of Bu_3SnH to 0.002 M (slow addition of Bu_3SnH by the syringe pump technique), the formation of **12b** was reduced (2%) and **11b** was obtained in 77% yield. Radical cyclizations to *gem*-difluoroolefin derivatives (**9a** and **9b**) also proceeded regioselectively in the exo mode to give **13a** and **13b**, respectively. Here, again, the high dilution method slightly improved the yield of 6-exo cyclization of **9b**.

Subsequently, the radical cyclization to α-fluoro-α,β-unsaturated lactone (**4**) was examined. Since the Emmons reaction of **6** exhibited the high E-selectivity (cis-relationship with respect to F and H), the cyclization system was easily prepared from the α-hydroxyaldehyde derivative (**14**). Deprotection-lactonization by treating **15** with *p*-TsOH in toluene-ethanol formed **16** as a diastereoisomeric mixture, which was then separated into less-polar and more-polar isomers (*l*- and *m*-**16**). Iodides (*l*- and *m*-**17**) derived from *l*- and *m*-**16** were subjected to radical cyclization, respectively. Reactions of *l*-**17** and *m*-**17** with Bu_3SnH gave bicyclolactones possessing the α-fluorine-substituent [*l*-**18** (95% yield) and *m*-**18** (54% yield), respectively].[8] By this approach to construct the bicyclolactone skeleton, a model study for the synthesis of fluorine-substituted Corey lactone was carried out. Retrosynthetically, the Corey lactone can be related back to the aldehyde derivative (**19**). The stereoselective Emmons reaction of **19** followed by deprotection of the acetal groups under acidic conditions gave the lactone (**20**). For cyclization via radical deoxygenation of the secondary hydroxyl group, **20** was converted to thiocarbonylimidazolide (**21**)[9] through pivaloylation. Tin hydride promoted the radical cyclization of **21** to give the bicyclolactone (**22**) in 43% yield as a stereoisomeric mixture,[10] which was a compound related to Corey lactone having α-fluorine substitution.

The present results clearly demonstrate the synthetic potential of fluoroolefins as radical acceptors in cyclization reactions. Fluoroolefination and radical cyclization conducted in conjunction is one means for synthesizing fluorine-containing ring compounds.

a) **6**, $NaN(SiMe_3)_2$/THF, 75%, b) *p*-TsOH/toluene-EtOH, 44%(*l*-) and 53%(*m*-), c) MsCl, Et_3N/CH_2Cl_2, then NaI/acetone, 91%(*l*-), 81%(*m*-), d) Bu_3SnH, cat. AIBN, benzene.

a) **6**, $NaN(SiMe_3)_2$/THF, 90%, b) HCl/toluene-EtOH, 55%, c) ^{t}BuCOCl/pyridine, 63%, d) $S{=}C(Imd)_2$/THF, 60%, e) Bu_3SnH, cat. AIBN, benzene, 43%.

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5) A mixture of two stereoisomers was obtained in each case except for **13a**; **10a** (2.9 : 1), **10b** (7.4 : 1), **11a** (1.3 : 1 by GLC), **11b** (1.6 : 1 in 0.02 M), **11b** (1.5 : 1 in 0.002 M), **13b** (2.2 : 1 in 0.04 M), **13b** (2 : 1 by GLC in 0.003 M). Cyclohexane derivatives (**10b** and **11b**) were trans isomers with respect to substituents on the ring. These were epimeric mixtures at the fluorine-substituted carbon atom.
6) Spectral data of the more polar isomer of **10a** (68%) as follows; ^{1}H-NMR δ: 1.03 (3H, t, J=7.2 Hz), 1.66-1.91 (4H, m), 2.00-2.19 (2H, m), 2.58-2.72 (1H, m), 3.08-3.15 (1H, m), 3.67 (1H, dq, J=10.8 and 7.2 Hz), 3.84 (1H, dq, J=10.8 and 7.2 Hz), 4.88 (1H, dd, J=48.8 and 3.5 Hz), 7.13-7.28 (5H, m). ^{19}F-NMR δ (from benzotrifluoride): -137.08 (dd, J=48.8 and 29.8 Hz). IR (neat): 1760 and 1739 cm^{-1}. High-resolution MS *m/z*: Calcd for $C_{15}H_{19}FO_2$: 250.1368. Found: 250.1375.
7) Compared with the cases of **8b** and **9b**, a high yield of the 6-exo cyclization of **7b** without high dilution conditions can be rationalized by preferencial attack of the nucleophilic alkyl radical to the electron-deficient olefin (α,β-unsaturated ester).
8) Ratio of stereoisomers; *l*-**18** (10 : 1), *m*-**18** (4.2 : 1). The major stereoisomers of *l*- and *m*-**18** were assigned based on the data of NOE experiments as shown below.
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10) In the present model study, stereochemical control is not discussed. Although (*R*)-glyceraldehyde-1,2-acetonide was used as the starting material for the synthesis of **19**, diastereomer separation for obtaining the single stereoisomer in chain-extension steps was difficult. **21** was a mixture of two diastereoisomers (2.3 : 1 by ^{1}H-NMR). The stereochemistry of **22**, a mixture of four stereoisomers (2.5 : 2 : 1.9 : 1), thus has yet to be fully clearified.

(Received July 9, 1991)

RATIONAL DESIGN AND SYNTHESIS OF A NOVEL CLASS OF ACTIVE SITE-TARGETED HIV PROTEASE INHIBITORS CONTAINING A HYDROXYMETHYLCARBONYL ISOSTERE. USE OF PHENYLNORSTATINE OR ALLOPHENYLNORSTATINE AS A TRANSITION-STATE MIMIC[1)]

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A novel class of HIV-1 protease inhibitors containing a hydroxymethylcarbonyl (HMC) isostere were designed from the substrate transition state and synthesized. Phenylnorstatine [Pns; (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] and the 2*S* diastereomer, (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, named allophenylnorstatine (Apns) were effective transition-state mimics, and incorporation of Pns-Pro or Apns-Pro at the P_1-P_1' site gave potent and specific HIV-1 protease inhibitors. In the inhibitory assays, the chemically synthesized [$Ala^{67,95}$] HIV-1 protease was used.

KEYWORDS HIV protease; HIV protease inhibitor; design; peptide synthesis; hydroxymethylcarbonyl isostere; transition-state mimic; phenylnorstatine; allophenylnorstatine; AIDS

The human immunodeficiency virus type-1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), codes for a virus-specific aspartic protease known to be essential for maturation and replication of the retrovirus. The HIV-1 protease is considered as a potential target for the development of agents for the treatment of AIDS and related diseases.[2)] Mammalian aspartic proteases such as renin and pepsin have two characteristic Asp-Thr-Gly sequences at the active center of the enzymes and both side chain carboxyl groups are important in the catalysis of the peptide bond cleavage. In contrast, the retroviral protease has only one Asp-Thr-Gly sequence and is presumed to function as a dimeric form. Proteases of HIV-1 and other retroviruses recognize the Xaa-Pro sequence as the cleavage site (Phe-Pro or Tyr-Pro for HIV-1 protease), but mammalian aspartic proteases do not. This feature provided a basis for the rational design of selective anti-AIDS agents.

Several groups reported HIV-1 protease inhibitors based on the transition-state analogue concept, containing a non-hydrolyzable bond, such as reduced amide,[3)] hydroxyethylene[4)] or hydroxyethylamine[5)] at the scissile peptide bond. Now, we report a novel class of HIV-1 protease inhibitors containing a hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic.

The transition state of amide hydrolysis by an aspartic protease is proposed as illustrated in Fig.1.[6)] The hydrogen bond between the carboxylic acid of protease and the hydroxyl group of the substrate transition state is very important in the design of tight-binding inhibitors. The HMC structure was reported to be effective in renin inhibitory potency.[6,7)] Therefore, we reasoned that the HMC group at the scissile site would interact at the active site of HIV-1 protease and the peptide compounds containing this structure (Fig.1) would be highly potent HIV-1 protease inhibitors.

In order to design substrate-based HIV protease inhibitors, we turned our attention to the p17/p24 cleavage site region (Fig.2).[8)] As described above, Tyr-Pro (or Phe-Pro) is a unique structure for HIV-1 protease and the synthetic substrate containing this sequence has low Km value.[9)] However, the phenolic hydroxyl group of the P_1 site and the side chain of Gln at the P_3 site seem to have no apparent effect on binding to HIV-1 protease.[10)] On the basis of these specifications, we

Fig.1. The Substrate Transition State in the Protease Active Site and the Hydroxymethylcarbonyl(HMC) Isostere

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..............Ser—Gln—Asn—Tyr—*—Pro—Ile—Val......... p17/p24 region

..............Ser—Phe—Asn—Phe—*—Pro—Gln—Ile......... TF / PR region

[H—Ser—Phe—Asn—Phe—Pro—Ile—Val—NH$_2$]

H—Ser—Phe—Asn—NH—(Pns, OH)—C(=O)—Pro(N)—CO—Ile—Val—NH$_2$

Fig.2. Design of Substrate-Based Inhibitors of HIV Protease

thought of a heptapeptide amide, Ser-Phe-Asn-Phe-Pro-Ile-Val-NH$_2$, similar to the TF/PR and p17/p24 sequences. Then, we incorporated an unnatural amino acid, phenylnorstatine[6] [Pns; (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid][11] containing the HMC isostere as a transition-state mimic at the P_1 site in this peptide amide (Fig.2).

Since symmetric-type inhibitors, based on the dimeric character of HIV-1 protease, were reported to be highly potent,[12] we also designed a symmetric-type inhibitor containing an HMC structure at the symmetric axis.

These peptides were synthesized by the solid-phase method based on Boc-Bzl strategy. Starting from MBHA•HCl resin (0.79 meq/g), each Boc-amino acid derivative was incorporated by the efficient method.[13] The protected peptide resin thus obtained was treated with anhydrous HF containing *m*-cresol at 0°C for 60 min. The RP-HPLC patterns of the crude peptides showed that these peptides were nearly pure, which means that the protection of the hydroxyl group of Pns was not necessary in this solid phase strategy. The crude peptides were purified by the FPLC system on an ODS-column. In addition, we synthesized several related peptide analogues by essentially the same method (Table I).

HIV-1 protease is formed from two identical 99 amino acid peptides by means of hydrophilic and hydrophobic interactions, not by a disulfide bond. Kent *et al.*[14] synthesized an HIV-1 protease analogue, in which the two Cys residues were replaced by the isosteric L-α-amino-n-butyric acid, whereas we replaced the two Cys residues by a natural amino acid, L-alanine. The chemically synthesized [Ala^{67,95}]-HIV-1 protease[15] had sufficient cleaving effect on the synthetic nonapeptide

Table I. Protease Inhibitory Activities (IC_{50})

No	Structure	HIV-1 protease	porcine pepsin[a]
1 (KNI-122)	H—Ser—Phe—Asn—NH—(Pns, OH)—C(=O)—Pro—Ile—Val—NH$_2$	100 nM	> 10,000 nM
1a (KNI-93)	H—Ser—Phe—Asn—NH—(Pns, OH)—C(=O)—Pro—Ile—Val—NH$_2$	5 nM	> 10,000 nM
2	PhCH$_2$CH$_2$CO—Asn—NH—(Pns, OH)—C(=O)—Pro—Ile—Val—NH$_2$	3,000 nM	> 10,000 nM
3	PhCH$_2$CH$_2$CO—Ser—NH—(Pns, OH)—C(=O)—Pro—Ile—Val—NH$_2$	>10,000 nM	N.D.[b]
4	PhCH$_2$CH$_2$CO—Asn—NH—(cyclohexyl analogue, OH)—C(=O)—Pro—Ile—Val—NH$_2$	>10,000 nM	N.D.[b]
5	H—Val—Val—NH—(Pns, OH)—C(=O)—Phe—Val—Val—NH$_2$	350 nM	4,000 nM
6	Ac—Phe—Asn—NH—CH(CH$_2$Ph)—CH$_2$—Pro—Gln—Ile—NH$_2$	>10,000 nM	N.D.[b]
Pepstatin A		933 nM	2,600 nM

[a] Albumin-BPB method[18] (pH 2.0). [b] Not determined.

substrate, Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2[3b] (pH 5.75, 37°C). We examined the protease inhibitory activities of the above peptidic compounds containing the HMC structure by this assay system.

Compound **1** (KNI-122) containing Pns as a transition-state mimic exhibited a potent inhibitory activity against HIV-1 protease as shown in Table I. Deletion of P_4 Ser and replacement of P_3 Phe by isosteric 3-phenylpropionic acid (compound **2**) also maintained sufficient inhibitory activity. However, replacement of P_2 (Asn→Ser,[16] compound **3**) or replacement of Pns by cyclohexylnorstatine[6,17] in P_1 (compound **4**) significantly decreased the inhibitory potency. The symmetric compound **5** showed potent inhibitory activity. The P_1 hydroxyl group was critical for the activity (compound **6**). Incorporation of (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid,[11] named allophenylnorstatine (Apns), the 2*S* diastereomer of Pns, as a transition-state mimic [compound **1a** (KNI-93)] surprisingly enhanced the inhibitory activity. Furtheremore, incorporation of (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA)[5a] at P_1 site [$Ph(CH_2)_2CO$-Ser-AHPPA-Pro-Ile-Val-NH_2] drastically decreased the inhibitory potency (IC_{50}>1,000,000nM; not shown in Table I) as expected from the additional carbon atom in the main chain.

Next, we examined the selectivity of these HIV-1 protease inhibitors against other aspartic proteases. As shown in Table I, compounds **1** (KNI-122), **1a** (KNI-93) and **2** did not practically inhibit pepsin, while the symmetric compound **5** showed a pepsin inhibitory activity equal to pepstatin A.

In conclusion, a novel class of HIV protease inhibitors were designed and synthesized using a "hydroxymethylcarbonyl (HMC) isostere" as a transition-state mimic. Incorporation of Pns-Pro or Apns-Pro at the P_1-P_1' site significantly enhanced the inhibitory potency and selectivity against HIV-1 protease. Thus, these compounds are good leads in the further design of selective anti-AIDS drugs.

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15) The [$Ala^{67,95}$] derivative (amino acid sequence: PQITLWQRPL [10]VTIKIGGQLK [20]EALL**DTG**ADD [30]TVLEEMNLPG[40]RWKPKMI-GGI[50]GGFIKVRQYD[60]QILIEI**A**GHK [70]AIGTVLVGPT [80]PVNIIGRNLL[90]TQIG **A**TLNF[99]) of HIV-1 protease (NY-5 isolate) was synthesized by the solid-phase method based on the Boc-Bzl strategy using PAM resin. Each Boc-amino acid derivative was incorporated by the efficient method[13] and the final deprotection was performed by the HF method. The product was purified by the usual method.[14] This replacement of Cys with Ala reduced the difficulties in the chemical synthesis and handling of the protease. In addition, this protease analogue could be prepared by the recombinant technique. The details of the synthesis will be reported elsewhere.
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(Received July 31, 1991)

 Chem. Pharm. Bull. 39(9) 2468—2470 (1991) Vol. 39, No. 9

ISOLATION OF A NEW ANTITUMOR SUBSTANCE FROM *BACILLUS STEAROTHERMOPHILUS*

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A new antitumor substance, BS-1, was isolated from the autolysate and culture filtrate of *Bacillus stearothermophilus* UK563 by ethylacetate extraction and HPLC. BS-1 inhibited the proliferation of mouse macrophage-like cells, P388-D1 (IC_{50}: 4 μg/ml) and mouse mastocytoma, P-815 (IC_{50}: 0.6 μg/ml), but not that of Balb/c 3T3.

KEYWORDS *Bacillus stearothermophilus*; thermophile; antitumor substance; P388-D1; P-815

Recently, great interest has developed in the application of thermostable enzymes produced by the thermophile for bioreactor, biosensor and clinical reagents, in addition to its uses in industrial technology.[1] Thermophile contains a variety of components such as lipids and polyamines which are chemically different from those of mesophile.[2] In connection with our studies on products of thermophile with potentially useful biological activity, we focused on *B. stearothermophilus* which has much short generation time (11min).[3] In this paper, we present the isolation of antitumor substance from the autolysate and culture filtrate of *B. stearothermophilus* UK563.

EXPERIMENTAL

Isolation *B. stearothermophilus* UK563 (Fermentation Research Institute, Deposit No. 7275) was cultured in a 500-liter continuous fermenter at 60°C at a flow rate of 400 l/h in a medium (adjusted to pH 7.0) of the following composition: glucose (0.35%), yeast extract (0.3%), pepton (0.1%), KH_2PO_4 (0.2%), Na_2HPO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.1%), $FeSO_4 \cdot 7H_2O$ (0.005%), $MnSO_4 \cdot H_2O$ (0.0001%) and Na_2MoO_4 (0.0001%).[4] After 4 days culture, the culture broth was separated into cells (approximately 50 g) and fluid (approximately 10 l) by centrifugation. The collected cells were autolyzed by incubating in 25 mM phosphate buffer (pH 8.0)-2 mM ethylenediaminetetraacetic acid at 40°C for 2 h. Cell debris was removed by centrifugation. The supernatant was concentrated to 1/5 volume *in vacuo* and extracted with ethylacetate. After removal of the solvent, the extract was dissolved in deionized water and activated ODS resin (Waters, 55-105 μm) was added. The ODS resin recovered by filtration on a glass filter was washed with 0.5% CH_3CN and eluted with 18% CH_3CN. The 18% CH_3CN fraction was evaporated *in vacuo*, and approximately 40 mg of oily residue containing the substance that inhibits the proliferation of mouse macrophage-like cells, P388-D1, was obtained from 1 kg of the mycelium. The oily residue of the 18% CH_3CN fraction was dissolved in 0.05% HCl and subjected to preparative HPLC on a Cosmosil $10C_{18}$ column. A sample of the column effluent was assayed for antitumor activity. An antitumor substance in the culture filtrate as well as the autolysate was concentrated by extraction with ethylacetate and treatment with ODS resin, and purified by HPLC on the ODS column.

Antitumor Activity Mouse mastocytoma, P-815, and P388-D1 were supplied by the Japanease Cancer Reseach Resources Bank. Culture medium was RPMI 1640 (Nissui) supplemented with 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % fetal calf serum. The cells (2×10^5/ml) were incubated with samples in 24-well multiplates (Sumilon) for 48 hr at 37 °C. A viability test was carried out by a trypan blue dye exclusion method.[5] Bleomycin A_2 (BLM) and 5-fluorouracil (5-FU) were used as positive controls.

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RESULTS AND DISCUSSION

An active component in the autolysate of the strain UK563 for inhibition of the proliferation of P388-D1 was purified by HPLC on Cosmosil $10C_{18}$ (Fig. 1a). The activity was recovered in a peak which eluted at 26.5 min. The homogeneity was

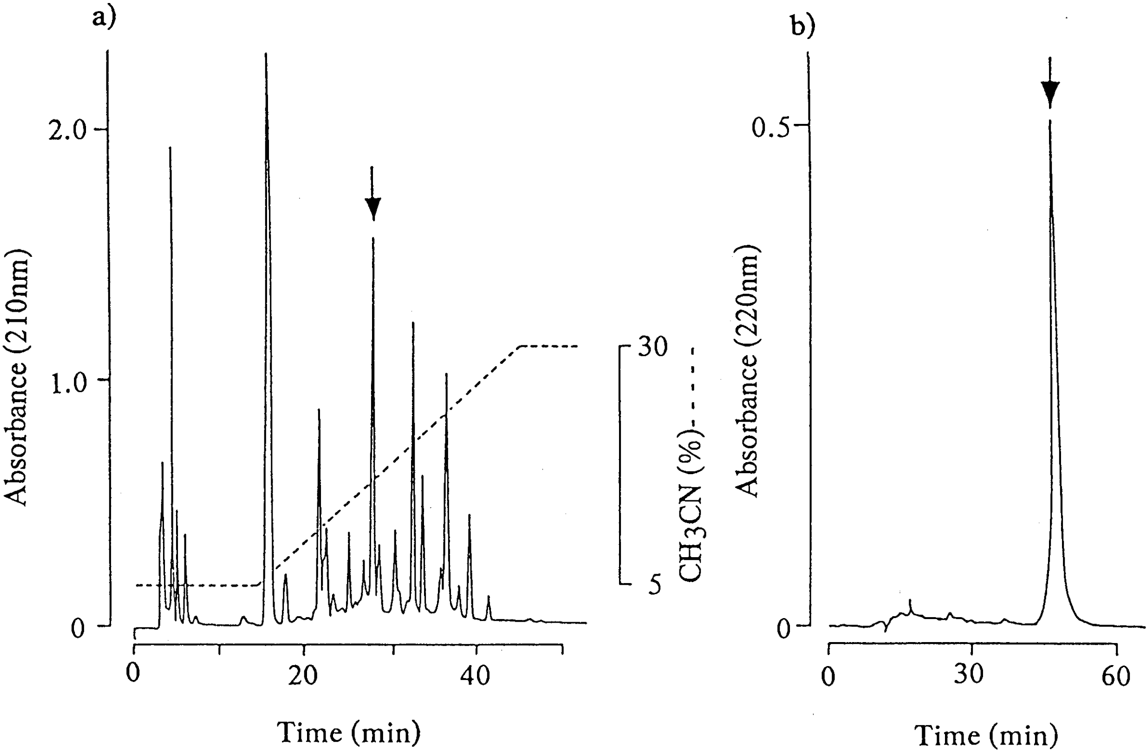

Fig. 1 Separation of Antitumor Substance on HPLC
Arrows indicate peaks with antitumor activity.
a) Column (Cosmosil $10C_{18}$): 0.46x25cm, solvent: 5-30 % CH_3CN in 0.05% HCl, flow rate: 1 ml/min, sample: 18% CH_3CN fraction in 100 μl of 0.05% HCl (equivalent to approximately 10 g of the mycelium).
b) Column (Asahipak GS-320): 0.76x50cm, solvent: 50 mM CH_3COONH_4 (pH6.7)/CH_3CN (90:10), flow rate: 1 ml/min, sample: active fraction from Cosmosil in 100μl of solvent.

confirmed by another HPLC of the final preparation on Cosmosil $5C_{18}$-AR (0.46x25cm) under the same condition as shown in Fig. 1a and on Asahipak GS-320 as shown in Fig. 1b. The final preparation was designated as BS-1. The antitumor activity in the culture filtrate of UK-563 was also derived from BS-1: because a single peak of absorbance co-eluting with antitumor activity in a final purification step showed the same retention time as that of BS-1. The yield of BS-1 was approximately 50 μg from each of the cells (1 kg) and the culture filtrate (200 l). Therefore, BS-1 must be a secondary metabolite of the strain

Table I. Antitumor Activity *in Vitro* of BS-1

Sample	Concentration (μg/ml)	Inhibition percent of proliferation		
		P-815	P-388-D1	Balb/c 3T3
BS-1	0.5	45	1	0
	1	66	5	0
	2.5	99	25	0
	10	—	87	0
	25	—	—	29
BLM	1.5	94	14	77
5-FU	0.01	63	10	21

UK563. BS-1 dose-dependently inhibited the proliferation of P388-D1 at doses ranging from 0.5 to 10 μg/ml (Table I), and its 50% inhibitory concentration (IC_{50}) was 4μg/ml. On the other hand, BS-1 inhibited the proliferation of mouse whole embryo, Balb/c 3T3 by 29% at 25 μg/ml. The ratio of the IC_{50} against Balb/c 3T3 to the IC_{50} against P388-D1 was more than 6. BS-1 also was markedly antitumoric against P-815 (IC_{50}:0.6 μg/ml) (Table I), and the ratio of IC_{50} against Balb/c 3T3 to IC_{50} against P-815 was more than 40. So far as we know, this is the first report with respect to an antitumor substance in products of thermophile. Physicochemical properties will be presented shortly. Further, the action mechanism and antitumor activity *in vivo* of BS-1 should be studied.

ACKNOWLEDGEMENT We are grateful to Nippon Kayaku Co.Ltd., for the supply of bleomycinA_2.

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(Received August 1, 1991)

DOUBLE DIELS-ALDER CYCLOADDITIONS OF 2(1*H*)-PYRIDONES ACTING AS DIENOPHILES

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The first examples are presented of double Diels-Alder cycloadditions used, as dienophiles, 2(1*H*)-pyridones, and of Diels-Alder cycloaddition of 1-unsubstituted 2(1*H*)-pyridone acting as a dienophile under atmospheric and high pressure conditions.

KEYWORDS double Diels-Alder cycloaddition; 1-substituted 2(1*H*)-pyridone; 1-unsubstituted 2(1*H*)-pyridone; dienophile; high pressure

2(1*H*)-Pyridones are classified as aromatic heterocycles, but the possibilities of the Diels-Alder cycloaddition of these compounds acting as dienes and dienophiles are of considerable interest in the syntheses of potentially valuable heterocyclic compounds. In contrast to the substantial amount of experimental work on the Diels-Alder cycloadditions of 2(1*H*)-pyridones acting as dienes,[1)] little attention has been focussed on similar reactions of these compounds acting as dienophiles.[2)] Here we report the double Diels-Alder cycloadditions of 1-substituted and 1-unsubstituted 6-acetyl-2(1*H*)-pyridones (**1a,b**) having two enone moieties with 2,3-dimethylbuta-1,3-diene[2)] (**2**) under atmospheric and high pressure conditions. Although the high pressure strategy[3)] has proven extremely useful to surmount the energy barrier imposed by the steric and electronic effects in cycloaddition reaction, such as Diels-Alder reaction, an application of the technique in Diels-Alder cycloadditions of 2(1*H*)-pyridones acting as dienophiles has not been reported.

These reactions are the first examples of the double Diels-Alder cycloadditions of 2(1*H*)-pyridones acting as dienophiles, and also of Diels-Alder cycloaddition of 1-unsubstituted 2(1*H*)-pyridone acting as dienophile.

Diels-Alder cycloaddition of 6-acetyl-1-methyl-2(1*H*)-pyridone (**1a**) with the diene (**2**) (5 eq.) was carried out under atmospheric (180°C, 96 h, toluene) or high pressure (10 Kbar, 80°C, 96 h, toluene) conditions, and the *cis-anti-cis* adduct (**3a**), mp 178-179°C, was stereoselectively obtained in 11% and 19% yields (72% and 60% yields of **1a** were recovered), respectively. The *cis-anti-cis* stereochemistry of **3a** was confirmed by X-ray analysis (Fig. 1).[4)]

Next, we examined Diels-Alder cycloaddition of 6-acetyl-2(1*H*)-pyridone (**1b**) with the diene (**2**) under the same reaction conditions. High pressure Diels-Alder cycloaddition of **1b** with **2** stereoselectively gave the *cis-anti-cis* adduct (**3b**), mp 174-175°C, $J_{6a,10a}$ 4.2Hz (400 MHz ^{1}H-NMR) in 11% yield (74% yield of **1b** was recovered). However, Diels-Alder cycloaddition of **1b** with **2** under atmospheric pressure conditions afforded a mixture of the *cis-anti-trans* adduct (**3c**), mp 185-186°C, $J_{6a,10a}$ 11.7Hz (400 MHz ^{1}H-NMR) in 10% yield, and the

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cis adduct (**4**), mp 77-79°C, $J_{4a,8a}$ 5.5Hz (400 MHz ^{1}H-NMR) in 6% yield, respectively. The yield of **1b** recovered was 54%.

The structure of **3b** was proved by the facile conversion to the 5-methyl adduct (**3a**) (96%) on treatment with methyl iodide (2 eq.) and KF-alumina[5] (*ca.* 3 eq.) in acetonitrile at room temperature. Thermal conversion of the *cis-anti-cis* adduct (**3b**) under the same atmospheric pressure conditions (180°C, 96 h, toluene) gave the *cis-anti-trans* adduct (**3c**) in 93% yield. Also, the reaction of the *cis* adduct (**4**) with **2** under the same high pressure conditions (10 Kbar, 80°C, 96 h, toluene) gave the *cis-anti-cis* adduct (**3b**) in 95% yield.

All new compounds, **3a-c** and **4** were characterized by IR and 400 MHz ^{1}H-NMR (^{1}H-^{1}H and ^{1}H-^{13}C COSY and NOE spectra) spectroscopy, and gave satisfactory elemental analyses.

Me N O O R

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

HH 10a 6a H N O Me-C O R

3a : R = Me, ~H = ◀H
3b : R = H, ~H = ◀H
3c : R = H, ~H = ---H

H 4a 8a H Me N O O H

4

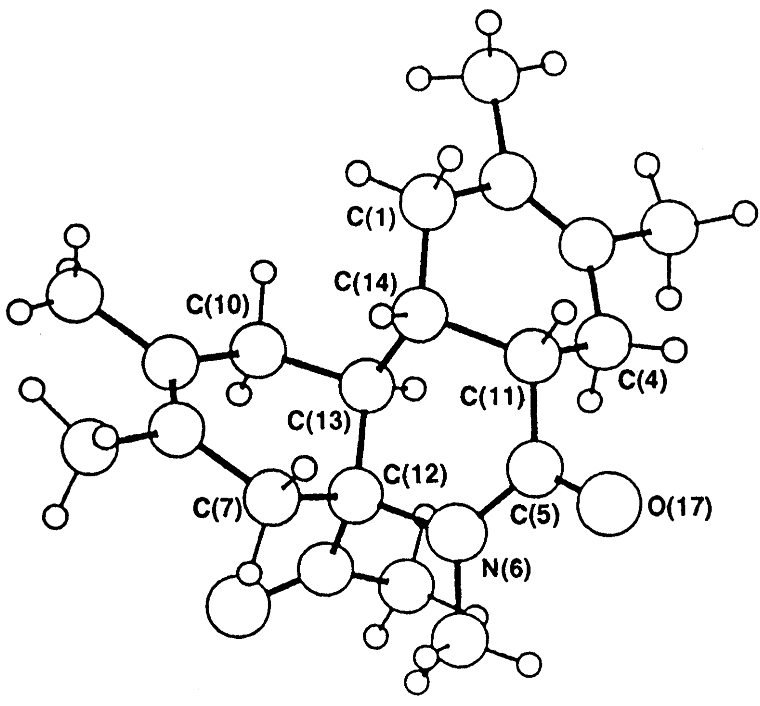

Fig. 1. Molecular Structure of *cis-anti-cis* Adduct (**3a**)

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4) Crystal data for **3a** : $C_{20}H_{29}NO_2$, M = 315.22, monoclinic, space group $P2_1/a$, a = 14.099(1), b = 22.139(2), c = 7.707(1)Å, α = 90.0(0)°, β = 129.69(1)°, γ = 90.0(0)°, U = 1852.2(3)$Å^3$, Z = 4, D_X = 1.131g/cm^3, F(000) = 688, μ(Cu-K_α) = 5.707cm^{-1}. λ(Cu-Kα) = 1.5418Å . 3234 unique reflections ($2\theta \geq 130°$) were measured , of which 2715 with $F \geq 2.67\sigma(F)$ were considered as observed. No absorption corrections were applied. The final R value is 0.058(Rw = 0.061).

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(Received August 2, 1991)