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Hydrolysis of Diphosphate Ion to Orthophosphate Ion in the Presence of Calcium Ion. Fundamental Study of the Development of the Mixed Crystal Deposition Disease

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The hydrolysis of diphosphate ion (pyrophosphate ion, PPi) to orthophosphate ion (Pi) in the presence of Ca^{2+} in aqueous systems were examined. The systems studied were (1) $K_4P_2P_7+CaCl_2$ with/without KOH, (2) $K_4P_2O_7+Ca(OH)_2$, (3) aqueous suspension of the precipitate isolated from the mother solution immediately after mixing of $K_4P_2O_7$ and $CaCl_2$, and (4) $K_4P_2O_7$ in an aqueous suspension of $Ca_2P_2O_7$. It was found that the gradual release of Ca^{2+} to the aqueous phase from the Ca^{2+}-reservoir (*i.e.*, $Ca(OH)_2$ formed (system 1) or added (system 2)) is effective for the hydrolysis of PPi. When the temporary precipitate containing PPi was suspended in the aqueous phase (system 3), the hydrolysis of PPi was delayed because re-dissolution of the precipitate was necessary prior to the hydrolysis. The result in system 4 suggested that the hydrolysis reaction occurs on the surface of $Ca_2P_2O_7$. These results are discussed in connection with a possible physicochemical mechanism of deposition of mixed crystals (calcium diphosphate and calcium orthophosphate) in the human body, causing mixed crystal deposition disease.

Keywords——hydroxyapatite; diphosphate hydrolysis; pyrophosphate hydrolysis; calcium diphosphate; calcium orthophosphate; crystal deposition disease; calcium pyrophosphate

Diphosphate ion (pyrophosphate ion, PPi) is present in body fluids such as blood, urine, and saliva. It has been suggested that PPi takes a role in preventing and/or regulating calcification of soft tissues and formation of undesirable concretion.[2] It is also known that the crystal growth and crystallite aggregation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) is retarded or inhibited by the presence of PPi in water.[3]

On the other hand, ectopic deposition of calcium salt of PPi ($Ca_2P_2O_7$, *i.e.*, Ca_2PPi) in arthrosis and yellow ligament causes pyrophosphate arthropathy (pseudo-gout syndrome)[4] and cervical radiculomyelopathy.[5] These diseases are associated with increased levels of PPi in the body fluids. Sometimes two kinds of calcium salts (*i.e.*, Ca_2PPi and HAP) are found in the affected part in crystal deposition diseases like these (mixed crystal deposition diseases).[6] Therefore, studies on the formation of these salts are physiologically and pathologically important.

In the previous papers,[7] it was shown that PPi is partially or totally hydrolyzed to orthophosphate ion (Pi) in the presence of Ca_2^+, forming amorphous calcium orthophos-

phate and/or HAP. The degree of crystallinity of HAP formed was higher in the presence[7a] than in the absence[7b] of alkaline substances (NH_4OH, for example). It was also found that hydrated calcium diphosphates prepared in an aqueous phase through the literature methods are contaminated with several kinds of by-products of orthophosphate salts.[7c] In the present paper, the transformation of the precipitates, which were formed temporarily immediately after mixing of $K_4P_2O_7$ with $CaCl_2$ (or $Ca(OH)_2$) in an aqueous phase, was studied as a function of time by considering re-dissolution of the precipitate, release of Ca^{2+}, K^+, and PPi from the precipitate, and/or the surface effect of the precipitate. A possible physico-chemical mechanism of deposition of mixed crystals (Ca_2PPi and HAP) in the human body is also discussed briefly.

Experimental

Materials——All chemicals used were of reagent grade from Nakarai Chemicals Ltd. or Wako Pure Chemical Industries Ltd. These were used without further purification. HAP was prepared as described elsewhere.[8] Crystalline calcium diphosphate ($Ca_2P_2O_7$, Ca_2PPi) was prepared by heating $CaHPO_4$ at 450 °C for 6 h, according to the method of McIntosh and Jablonsky.[9]

Preparative Methods——$MeCl_2$(Me = Mg^{2+}, Ca^{2+}, or Sr^{2+}) was added to 112.5 mM $K_4P_2O_7$(K_4PPi) in the presence or absence of 1 M KOH at 30 °C. The solution containing the precipitate formed was filtered through a 0.22 μm Millipore filter after incubation for 2 h. The contents of total phosphorus (P_t) and PPi in the filtered precipitates were analyzed.

A known weight of $Ca(OH)_2$ was added to an aqueous solution of 0.5 M K_4PPi (25 ml, pH 9.85) at 30 °C, resulting in the formation of a suspension. The pH immediately after mixing was 13.5—13.9, depending on the amount of $Ca(OH)_2$ added. The suspension was filtered through a 0.22 μm Millipore filter after various time intervals. The filtrate and the filtered precipitate were analyzed.

An aqueous solution of $CaCl_2$ (2M, 1.5 l) was added to 1.5 M K_4PPi (1 l), where the molar ratio of Ca^{2+} to PPi is 2.0, under vigorous stirring at 30 °C to obtain a precipitate immediately after mixing. The precipitate obtained within 5 min after the reaction was washed with water and methanol, air-dried, and finally dried *in vacuo* at room temperature. It was a white fine powder and was amorphous as judged by X-ray powder diffractometry. Its composition was determined to be $Ca_{1.53}K_{1.06}(P_2O_7)_{0.94}(PO_4)_{0.12}\cdot 4.0H_2O$ (precipitate 1) by means of chemical analysis. The precipitate 1 was suspended in various kinds of media (water, 4 M KCl, and 2 M $CaCl_2$) and incubated at 30 °C. The suspension thus obtained were filtered at various time intervals, and the filtrates and the remaining precipitates were analyzed.

Analysis——Immediately before the chemical analysis, the precipitates were dissolved quickly in an aqueous solution of ethylenediaminetetraacetate (EDTA). Calcium content was determined by measuring the excess EDTA remaining in the solution by chelatometry with a standard solution of $MgCl_2$ at pH 10 with BT indicator (1-(1-hydroxy-2-naphthylazo)-6-nitro-2-naphthol-4-sulfonic acid). Potassium content was determined by flame photometry (Hitachi model 205). The content of PPi was determined according to the method of Kato *et al.*[10] Zinc diphosphate (Zn_2PPi), precipitated from the sample solution by addition of an excess amount of zinc acetate at pH 3.8—3.9, was dissolved in NH_4Cl–NH_4OH buffer solution at pH 10. This solution, containing zinc ion ($[Zn^{2+}] = 2[PPi]$), was analyzed by chelatometry with EDTA standard solution and BT indicator at pH 10. It was confirmed that neither Ca^{2+} nor Pi, which is contained in the sample precipitate and/or filtrate, interferes with the titration, because these ions were excluded through the above procedure.

Content of P_t, which is present as Pi and PPi, was determined by colorimetry according to the method of Gee *et al.*[11] after hydrolysis of PPi to Pi by boiling the sample in 0.5 M HCl for 30 min. The phosphate ammonium molybdate complex formed was reduced with stannous chloride. The absorbance of the resulting color after 15 min was determined at 720 nm in a Shimadzu model UV-180 spectrophotometer. The concentration of Pi was determined as the difference between the content of total phosphorus ($[P_t] = [Pi] + 2[PPi]$) and that of PPi (2[PPi]).

X-Ray Diffraction——The X-ray powder diffraction patterns (Co K_α radiation at 35 kV and 10 mA with an iron filter, wave length 1.789 Å) were obtained with a Norelco Geiger Counter diffractometer (Philips Electronics and Pharmaceutical Industry) at room temperature.

Henceforth, subscript "l" or "s" indicates liquid phase (filtrate) or solid phase (precipitate).

Results

Effects of Cationic Species and OH^- on Hydrolysis of PPi

The effects of species of divalent metal cations (Me = Mg^{2+}, Ca^{2+}, and Sr^{2+}) and of the

mixing ratio of Me to PPi on the hydrolysis of PPi were examined in the presence and absence of 1 M KOH.

Chemical analyses after incubation for 2 h showed that PPi was not hydrolyzed to Pi in neutral or alkaline solution in the absence of Me. In the presence of Me, PPi was hydrolyzed to Pi, and the content of Pi in the precipitate increased with the molar ratio of Me to PPi because Me catalyzes the hydrolysis of PPi to Pi. The extent of hydrolysis was larger in the presence of OH^- than in the absence of OH^-, and in the presence of OH^- and Ca^{2+} (or Sr^{2+}) than in the presence of OH^- and Mg^{2+}. The molar ratio of Pi to P_t (Pi/P_t), as a parameter of the extent of hydrolysis, in the precipitate became almost unity when OH^- (as KOH) was added and the initial molar ratio of Me/PPi (Me=Ca^{2+} or Sr^{2+}) was higher than 3.0. However, the molar ratio of Pi/P_t was less than 0.4 even though Mg^{2+}/PPi was higher than 4.0 in the presence of KOH. The presence of $Ca(OH)_2$ was confirmed by X-ray powder diffractometry when KOH was added to the system containing Ca^{2+}. Crystalline $Sr(OH)_2$ or $Mg(OH)_2$ was not detected by X-ray powder diffractometry. However, amorphous $Sr(OH)_2$ or $Mg(OH)_2$ should have precipitated in the system containing Sr^{2+} or Mg^{2+} and OH^-. The reason why the extent of hydrolysis is smaller in the presence of $Mg(OH)_2$ than in the presence of $Ca(OH)_2$ or $Sr(OH)_2$ may be lower solubility of $Mg(OH)_2$ than of $Ca(OH)_2$ or $Sr(OH)_2$ (see Discussion also).

Hydrolysis of PPi in the Presence of $Ca(OH)_2$

After the mixing of 0.5 M K_4PPi (25 ml) and $Ca(OH)_2$ (1.25 g), the filtrate and the precipitate were analyzed chemically at various time intervals. It was found that the concentration of P_t in the filtrate decreased and the content of P_t in the precipitate increased with time. On the other hand, a small amount of Pi appeared in the filtrate, while the content of Pi in the precipitate increased gradually with time. These results show that PPi in the solution was hydrolyzed to Pi in the presence of Ca^{2+},[7] which was supplied gradually through the dissolution of $Ca(OH)_2$ added and was consumed by the precipitate formation with PPi and Pi formed. Thus, the hydrolysis of PPi and precipitate formation continued for more than 100 h.

The above analytical values were converted to molar ratio of Pi to P_t. Figure 1 shows the time courses of the molar ratios of Pi to P_t, Pi/P_t, in the filtrate, in the precipitate, and in the

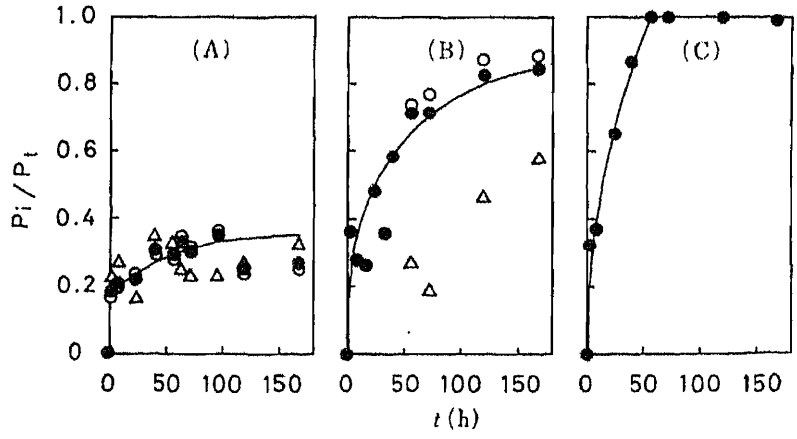

Fig. 1. Molar Ratio of Orthophosphate Ion to Total Phosphorus

Initially 1.25 g (A), 2.5 g (B), or 5.0 g (C) of $Ca(OH)_2$ was added to 0.5 M K_4PPi (25 ml). The initial molar ratio of Ca^{2+} to total phosphorus, Ca^{2+}/P_t, was 0.675 (A), 1.35 (B), or 2.7 (C), respectively. The concentrations of Pi and P_t in the filtrate of system (B) were too low to determine accurately. Neither Pi nor PPi were detected in the filtrate of system (C).

△, in the filtrate; ○, in the precipitate; ●, for the total system (the filtrate+the precipitate).

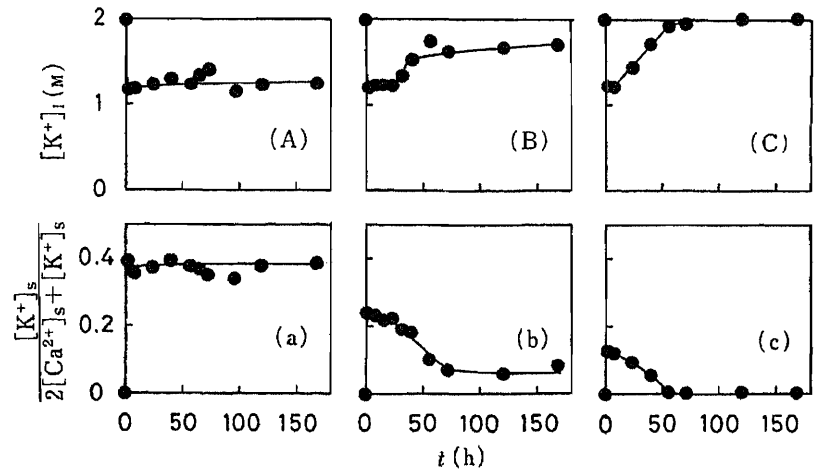

Fig. 2. Concentration of Potassium Ion in the Filtrate and Equivalent Fraction of Potassium Ion with Respect to Cations in the Precipitate

The initial molar ratio of mixing of Ca^{2+} to P_t, Ca^{2+}/P_t, was 0.675 ((A) and (a)), 1.35 ((B) and (b)), or 2.7 ((C) and (c)), as in Fig. 1. The equivalent fraction (not shown) of Ca^{2+} in the precipitate increased with time synchronously with the decrease in K^+ content.

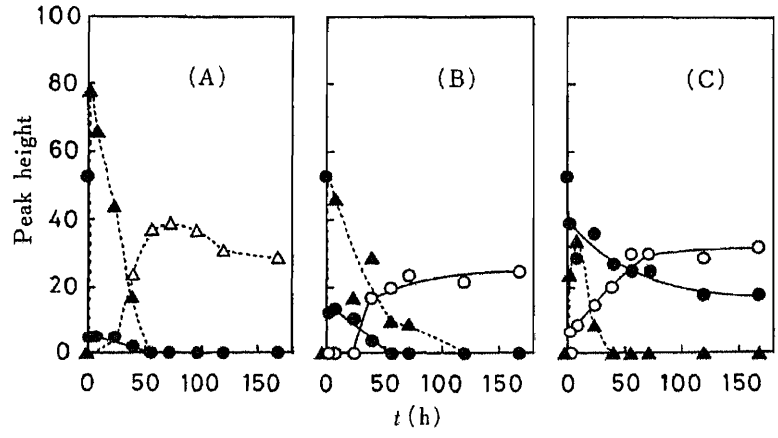

Fig. 3. Time Courses of the Peak Heights in X-Ray Powder Diffraction

The initial molar ratio of Ca^{2+} to P_t, Ca^{2+}/P_t, was the same as in Fig. 1.
—●—, $Ca(OH)_2$ (spacing d=1.93 Å)[13]; —○—, HAP (d=2.81 Å)[8];
---▲---, $CaK_2P_2O_7 \cdot 4H_2O$ (d=2.89 Å)[12]; ---△---, $CaK_2P_2O_7$ (d=2.18 Å).[12]

total system. The values of Pi/P_t in each phase increased with time. The initial slope in the figure became steeper with increase in the initial mixing ratio of Ca^{2+}/P_t. When the ratio, Ca^{2+}/P_t, was high enough (Fig. 1(C), for example), PPi was completely exhausted from the system and the ratio, Pi/P_t, became unity within a short period.

Figure 2 shows the time courses of the potassium concentration ($[K^+]_l$) in the filtrate ((A), (B), and (C)) and those of the equivalent fraction of potassium with respect to cations in the precipitate ($[K^+]_s/(2[Ca^{2+}]_s+[K^+]_s)$, (a), (b), and (c)). When the initial ratio, Ca^{2+}/P_t, was low ((A) and (a), for example), K^+ was captured in the precipitate and the level of K^+ in the filtrate decreased immediately after mixing of the reagents. However, when the ratio, Ca^{2+}/P_t, was high enough, K^+ was released from the temporary precipitate containing much K^+. That is, $[K^+]_l$ initially decreased and then increased ((B) and (C)), while the equivalent fraction of K^+ in the precipitate initially increased and then decreased ((b) and (c)) with time. Especially in the cases of (C) and (c), the figures suggest that K^+ was excluded completely from the

precipitate within 75 h and that the only cationic species in the precipitate was Ca^{2+}. Acidic salt containing H^+ would not appear because the pH of the mother solution was high enough (13.5—13.9).

Figure 3 shows the time courses of the diffraction intensity determined by X-ray powder diffractometry of the crystalline substances found in the precipitate.[12,13] The intensity of the diffraction by $Ca(OH)_2$ decreased gradually for about 50 h ((A) and (B)) or more than 50 h (C), depending on the amount of $Ca(OH)_2$ added, after a steep decrease immediately after mixing of the reagents. These results show that the dissolution and/or consumption of $Ca(OH)_2$ is gradual, as expected.

The species $CaK_2P_2O_7 \cdot 4H_2O$ was present for a while. However, $CaK_2P_2O_7$ (Fig. 3(A)) or HAP (Fig. 3(B) and (C)), both of which are more stable than $CaK_2P_2O_7 \cdot 4H_2O$,[12] appeared later synchronously with the disappearance of $CaK_2P_2O_7 \cdot 4H_2O$. The diffraction peak of HAP appeared earlier and the diffraction was stronger in (C) than in (B). These results may be due to higher contents of Pi, Ca^{2+}, and OH^- in the system shown in (C) than in the system shown in (B).

Transformation of the Precipitate Formed Temporarily

The amorphous precipitate **1** (5 g), prepared according to the method mentioned in Experimental, was suspended in 25 ml of water, 4 M KCl, or 2 M $CaCl_2$ to examine its transformation to a stable form in contact with an aqueous phase.

Partial dissolution and change of the chemical composition of the precipitate were suggested by the fact that the concentrations of P_t and K^+ in the supernatant increased and the total amount of P_t in the precipitate decreased with time when the precipitate **1** was soaked in water or in 4 M KCl. However, Ca^{2+} was not detected in the supernatant. On the other hand, when the precipitate **1** was suspended in 2 M $CaCl_2$, the concentration of K^+ increased and that of Ca^{2+} decreased in the supernatant with time. This means that the K^+ content decreased and the Ca^{2+} content increased in the precipitate through the absorption of Ca^{2+} from the medium.

The ratios of Pi to P_t, Pi/P_t, in the supernatant, in the precipitate, and in the total system (solution phase + precipitate phase) are shown in Fig. 4 as functions of the incubation time. The molar ratio of Pi/P_t increased with time irrespective of the suspending medium. The ratio Pi/P_t in 2 M $CaCl_2$ (C) is higher than that in water (A) and in 4 M KCl (B). This result shows

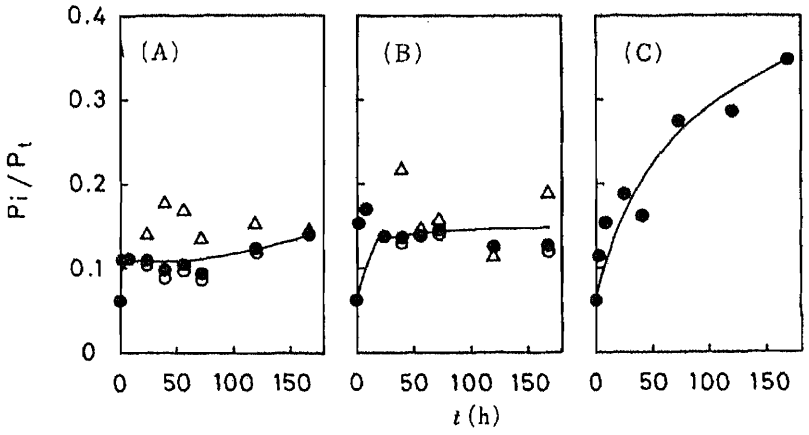

Fig. 4. Molar Ratio of Pi to P_t as a Function of Time

The precipitate 1 (5 g) was suspended in 25 ml of water (A), 4 M KCl (B), or 2 M $CaCl_2$ (C). The initial pH was adjusted to 7.0. Neither Pi nor P_t was detected in the filtrate of system (C).

△, molar ratio in the filtrate; ○, molar ratio in the precipitate; ●, molar ratio with respect to the total system (the filtrate + the precipitate).

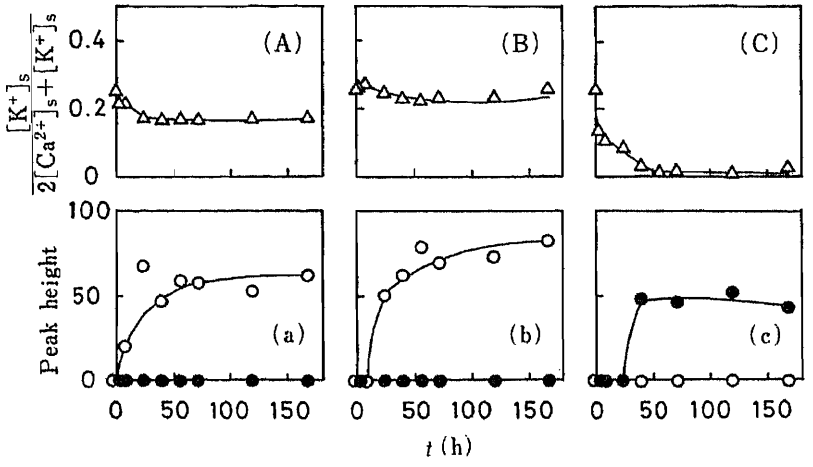

Fig. 5. Equivalent Fraction of Potassium Ion in the Precipitate and Peak Heights in X-Ray Powder Diffraction

The precipitate I (5 g) was suspended in 25 ml of water ((A) and (a)), 4 M KCl ((B) and (b)), or 2 M $CaCl_2$ ((C) and (c)).

△, equivalent fraction of K^+ in the precipitate; ○, peak height in X-ray powder diffraction of crystallites of $Ca_5K_2(P_2O_7)_3 \cdot 6H_2O$ (d=2.82 Å)[12]; ●, peak height in diffraction of crystallites of $Ca_2P_2O_7 \cdot 2H_2O$ (monoclinic, d=3.03 Å).[12]

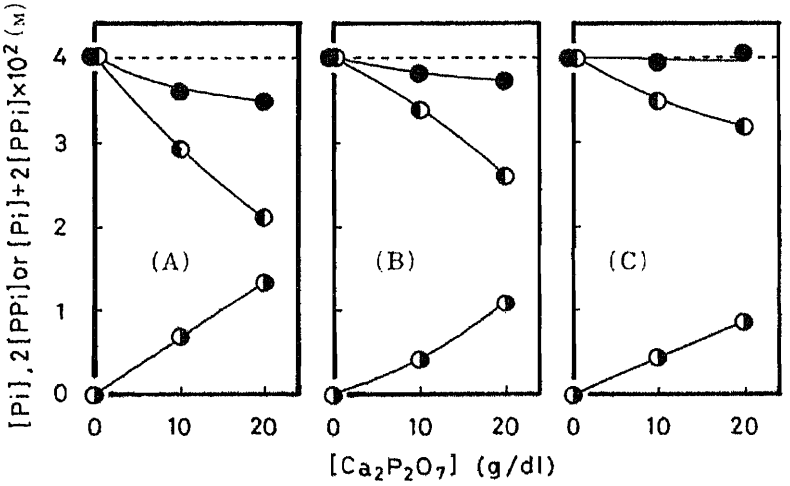

Fig. 6. Hydrolysis of PPi in the Presence of Ca_2PPi

A known amount of Ca_2PPi was suspended in 20 mM K_4PPi, and the initial pH was adjusted to 7 (A), 9 (B), or 13 (C) by the addition of HCl or KOH. The filtrate was analyzed after incubation for 72 h at 30 °C. The horizontal dotted line shows 40 mM, which is the total concentration of phosphorus added as 20 mM K_4PPi.

◑, [Pi]; ◐, 2[PPi]; ●, [Pi]+2[PPi].

that PPi is easily hydrolyzed to Pi in the presence of Ca^{2+}.[7] The data for the liquid phase are not shown in Fig. 4(C), because the concentrations of Pi and PPi in the solution were too low to be determined precisely due to excess Ca^{2+} in the solution.

Figures 5(A), (B), and (C) show the equivalent fractions of K^+ with respect to cations in the precipitate as functions of the incubation time. Simultaneously with the rearrangement of cations and anions in the precipitate, as mentioned above, crystallites of $Ca_5K_2(P_2O_7)_3 \cdot 6H_2O$ or $Ca_2P_2O_7 \cdot 2H_2O$ appeared and the diffraction intensity in X-ray powder diffractometry increased with time (Figs. 5(a), (b), and (c)). Molar ratios of K^+ to Ca^{2+} in these crystalline substances (0.40 for the former and 0.00 for the latter) became less than the initial molar ratio in the precipitate I (=0.69) owing to the decrease in the K^+ content in the precipitate.

Crystallites of $Ca_5K_2(P_2O_7)_3 \cdot 6H_2O$ were not found in the precipitate treated in 2 M $CaCl_2$, probably because almost all K^+ in the precipitate was extracted into the aqueous phase and Ca^{2+} was absorbed into the precipitate. In contrast to the result shown in Fig. 3, HAP crystallites were not detected in the precipitate by means of X-ray powder diffractometry, although the precipitate contains Pi and Ca^{2+}. This may be because of an insufficient quantity of OH^- in the mother solution (almost neutral) and because PPi, an inhibitor of HAP crystallization, is still present in the system.

The Effect of the Amount of Ca_2PPi Added and pH on the Extent of Hydrolysis of PPi

It was found that PPi in the aqueous phase was hydrolyzed to Pi to an extent depending on the added amount of Ca_2PPi (which is practically insoluble in water), and on the pH of the solution (Fig. 6). The concentration of Pi, formed by hydrolysis of PPi during the incubation for 72 h at 30 °C, increased with increase in the amount of Ca_2PPi added, $[Ca_2P_2O_7]$, and with decrease in the pH of the mother solution. Concomitantly, PPi remaining in the solution decreased with increase in $[Ca_2P_2O_7]$ and with decrease in pH. The closed circles, which show the concentration of total phosphorus ([Pi]+2[PPi]) in the solution, should be on the dotted line if these anions were not adsorbed by added Ca_2PPi. The figure, however, shows that adsorption occurs, and the extent of adsorption decreases with increase in pH owing mainly to the competitive adsorption of OH^- with Pi and PPi.

Discussion

The mechanism of hydrolysis and precipitate formation is considered as follows, taking into account the experimental results. An amorphous precipitate is formed immediately after mixing of PPi and Ca^{2+}; this precipitate contains Ca^{2+}, K^+, PPi, and Pi, the last-mentioned of which is formed through the hydrolysis of PPi. This precipitate crystallizes to a stable form synchronously with release of K^+ and PPi. The released PPi is also hydrolyzed to Pi in the presence of the catalyst Ca^{2+}, which is supplied through the gradual dissolution of $Ca(OH)_2$ as a Ca^{2+}-reservoir or which remains in the solution as excess $CaCl_2$. Therefore, the Pi content in the precipitate increases with time through the re-precipitation of Pi with Ca^{2+}. When an excess amount of Ca^{2+} with respect to PPi is present in the system, the hydrolysis may continue until the complete exhaustion of PPi, forming a precipitate of calcium orthophosphate. On the other hand, when extra Ca^{2+} is not supplied to the system, the amount of P_i in the precipitate decreases because PPi released from the precipitate and/or Pi formed from PPi cannot re-precipitate without Ca^{2+}. HAP crystallites appear in the precipitate when enough OH^- is present (Fig. 3). However, HAP was not detected in the system shown in Fig. 5, where OH^- was not supplied. HAP crystallites may appear after long-term ageing and/or ripening because the most stable form of calcium orthophosphate is HAP when the pH of the mother solution is higher than *ca.* 4.5.[14]

The hydrolysis proceeds through another mechanism, being accelerated by the adsorption of PPi on the surface of Ca_2PPi (Fig. 6).[7a] Therefore, it is reasonable to say that the dependence of the extent of hydrolysis on the OH^- concentration (or pH) is different under the conditions of Figs. 1—3 from that under the conditions of Fig. 6.

Calculus formation in the human body, as well as the hydrolysis and precipitate formation mentioned in the present paper, is the result of a rate process, not an equilibrium process. Therefore, the results mentioned above may be relevant to the mechanism of development of mixed crystal deposition disease,[6] although the experimental conditions here were not strictly physiological. The surface of the calculi, which are present in the affected part, and calcium ion, which is supplied continuously from the body fluids as a Ca^{2+}-reservoir (instead of $Ca(OH)_2$ or $CaCl_2$ added), may take part in the hydrolysis of PPi in the tissues and

in the body fluids in a similar manner to that mentioned in the text. Of course, the hydrolysis of PPi is catalyzed mainly by the enzyme pyrophosphatase. The PPi and Pi formed, in addition to Pi contained originally in the body fluids, precipitate together with Ca^{2+} on the matrices, and the mixed mineral deposit grows as calculi with time.

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Effect of Added Salt on the Adsorption of Dodecyl Sulfate Ion and Concurrent Release of Phosphate and Calcium Ions at the Surface of Hydroxyapatite

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Concentrations of phosphate ion ($[Pi]_f$) and calcium ion ($[Ca^{2+}]_f$) liberated from the surface of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAP) during the adsorption of dodecyl sulfate ion (DS^-) onto HAP were determined at various concentrations of added salt (NaCl or Na_2HPO_4). The effects of added salt on the relationships among $[Pi]_f$, $[Ca^{2+}]_f$, the concentration of DS^- ($[DS^-]_f$), and the adsorbed amount of DS^- (X_{DS^-}) are discussed. The amount of X_{DS^-} increased monotonously with the concentration of NaCl added ($[NaCl]_i$). However, the equilibrium concentrations of phosphate ion (Pi) and calcium ion (Ca^{2+}) changed in a complex manner with $[NaCl]_i$. It was concluded that ions on the surface of HAP were partly ion-exchanged with those of sodium dodecyl sulfate and NaCl, and that Ca^{2+} and Na^+ were bound competitively as counter-ions to DS^- micelles. On the other hand, when Na_2HPO_4 was added to the solution, added Pi (*i.e.*, one of the lattice ions for HAP) had a drastic effect on X_{DS^-} and $[Ca^{2+}]_f$. The adsorbed amount of DS^- decreased with increasing concentration of added Na_2HPO_4 owing to competitive adsorption between DS^- and added Pi. The equilibrium concentration of Ca^{2+}, $[Ca^{2+}]_f$, decreased for two reasons. First, adsorbed Pi inhibits the release of Ca^{2+} from the surface of HAP by virtue of the electrostatic attractive force. Secondly, the excess Pi remaining in the solution prevents Ca^{2+}-release from the surface of HAP since the solubility product for HAP must remain constant.

Keywords——hydroxyapatite; adsorption; dodecyl sulfate ion; phosphate ion release; calcium ion release; ion-exchange; micelle; solubility product; competitive adsorption; phosphate ion

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAP) is the main mineral component of mammalian hard tissues (teeth and bones) and human renal calculi. Thus, it is important to study the influence of organic ions on the surface properties of HAP in order to understand the processes occurring where the hard tissues contact body fluids and/or organic matrices in the human body.

In a previous study,[2] the influence of dodecyl sulfate ion (DS^-) on the HAP surface was studied at a constant mixing ratio of HAP to an aqueous solution of sodium dodecyl sulfate (SDS). It was found that phosphate ion (Pi) is released from HAP mainly by the mechanism of ion-exchange with DS^- adsorbed on HAP, whereas calcium ion (Ca^{2+}) is released mainly through its binding to DS^- micelles in the aqueous phase. The concentrations of released Pi and Ca^{2+} are restricted so as to keep the solubility product for HAP constant. Furthermore, the amount of DS^- adsorbed on the HAP surface and the amounts of Pi and Ca^{2+} released from the surface are also mutually balanced so as to maintain the electroneutrality of the surface phase of HAP.

In the present work, the effect of added salt (NaCl or Na_2HPO_4) on the adsorbed amount of DS^- and on the released amounts of the constituent ions of HAP (Pi and Ca^{2+}) was studied. The results are discussed, taking into account the competitive binding of counter-ions (Ca^{2+} and Na^+) to DS^- micelles, and competitive adsorption of DS^- and Pi on the surface of HAP.

Experimental

Materials——HAP and SDS were the same samples as those used previously.[2] Sodium chloride (NaCl) purchased from Nakarai Chemicals Ltd. was of reagent grade and was used after heating at 600 °C for 5 h to remove contaminating hydrochloric acid. Disodium hydrogen phosphate (Na_2HPO_4), purchased from Wako Pure Chemical Industries Ltd., was of reagent grade and was used without further purification.

Methods——HAP (0.7 g) was suspended in an SDS solution (35 ml) of a known concentration at 30 °C with or without added salt (NaCl or Na_2HPO_4), and the suspension was shaken vigorously at frequent intervals. No buffer solutions were used in order to avoid effects of buffering agents on the properties of the HAP surface and DS^- micelles. After at least 4 d, which was a sufficient time to reach adsorption and dissolution equilibria, the suspension was filtered through a Millipore filter (0.1 μm pore size), and the filtrate was used for chemical analyses.

The concentrations of dodecyl sulfate ion, phosphate ion, and calcium ion were determined by the same methods as before.[2] The adsorbed amounts of DS^- and Pi were calculated from the difference of the concentrations before and after addition of HAP. The pH of the filtrate was measured on a Toa HM-5ES pH meter.

Results

Adsorption Isotherms of DS^- on HAP

Figures 1 and 2 show the adsorption isotherms of DS^- on HAP from an aqueous solution of SDS at constant concentrations of added NaCl (Fig. 1) and Na_2HPO_4 (Fig. 2). As shown in Fig. 1, the adsorbed amount of DS^- (X_{DS^-}) increased to a maximum, and then decreased with increasing concentraion of free DS^- ($[DS^-]_f$). It was found that X_{DS^-} increased with increasing concentration of added NaCl ($[NaCl]_i$), and that the negative slope at high $[DS^-]_f$ became less steep as $[NaCl]_i$ increased.

On the other hand, when Na_2HPO_4 was added to an SDS solution (Fig. 2), X_{DS^-} decreased with increasing concentration of added Na_2HPO_4 ($[Na_2HPO_4]_i$) except in the region of low $[DS^-]_f$, where X_{DS^-} at $[Na_2HPO_4]_i = 13.6$ mM was larger than that at $[Na_2HPO_4]_i = 2.73$ mM.

Concentration of Pi and Equilibrium pH of the Solution

Figure 3A shows the relationship between $[DS^-]_f$ and the concentration of phosphate ion released from the surface of HAP ($[Pi]_f$) in the presence of added NaCl. It was found that $[Pi]_f$ increased with $[DS^-]_f$. When $[DS^-]_f$ was low, $[Pi]_f$ increased with increasing $[NaCl]_i$, that is, in the order of $[NaCl]_i = 0$, 25, 100 and 450 mM. This order, however, was reversed gradually as $[DS^-]_f$ increased. When $[DS^-]_f$ became higher than 5.0 mM, $[Pi]_f$ decreased with increasing

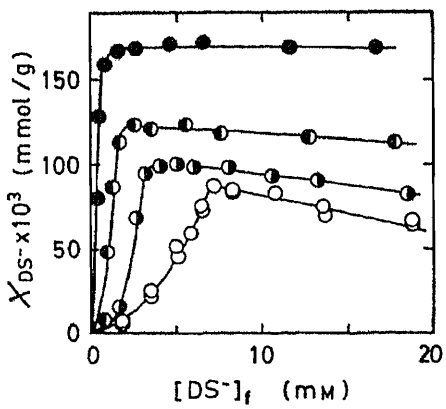

Fig. 1. Adsorption Isotherms of DS^- on HAP from Aqueous Solution of SDS at Constant Initial Concentration of Added NaCl

Initial concentration of added NaCl, $[NaCl]_i$ (mM): ○, 0; ◑, 25; ◐, 100; ●, 450.

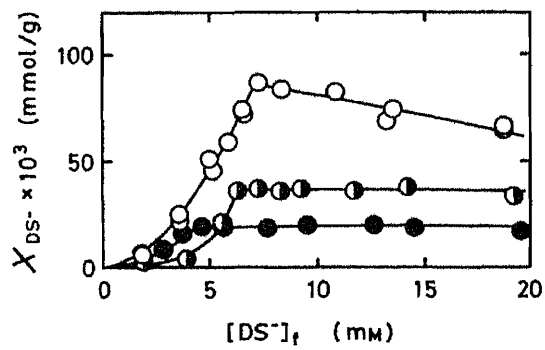

Fig. 2. Adsorption Isotherms of DS^- on HAP from Aqueous Solution of SDS at Constant Initial Concentration of Added Na_2HPO_4

Initial concentration of added Na_2HPO_4, $[Na_2HPO_4]_i$ (mM): ○, 0; ◑, 2.73; ●, 13.6.

$[NaCl]_i$. The equilibrium pH, $(pH)_f$, of the solution is plotted against $[DS^-]_f$ in Fig. 3B. It was found that $(pH)_f$ increased with increasing $[DS^-]_f$, and the order of the effect of $[NaCl]_i$ was almost the same as that shown in Fig. 3A. This result shows that the increase of $(pH)_f$ is closely related to the protonation of the phosphate ion released into the solution.

Figure 4 shows the relationship between $[Pi]_f$ and $[DS^-]_f$ at constant concentration of added Na_2HPO_4. When $[Na_2HPO_4]_i$ was 0 mM (C) or 2.73 mM (B), $[Pi]_f$ increased with $[DS^-]_f$ by virtue of Pi-release from HAP. On the other hand, when $[Na_2HPO_4]_i$ was 13.6 mM (A), $[Pi]_f$ became less than $[Na_2HPO_4]_i$ owing to the adsorption of Pi. The equilibrium pH of the solution was almost constant (7.65—7.70 for $[Na_2HPO_4]_i = 2.73$ mM, 8.17—8.23 for $[Na_2HPO_4]_i = 13.6$ mM) irrespective of $[DS^-]_f$ due to the buffering function of Pi when enough Na_2HPO_4 was added (compare with the data in the absence of the added salt (○ in Fig. 3B).

Concentration of Ca^{2+} Released from the Surface of HAP

Figure 5 shows the relationship between $[DS^-]_f$ and the concentration of calcium ion released from the surface of HAP ($[Ca^{2+}]_f$) at constant concentration of added NaCl. The curve consists of two branches: one is downward and another upward, and the slope of the latter decreases with increasing $[NaCl]_i$. The concentration of DS^- at the break point in Fig. 5 (6.7, 3.2, 2.0 and 1.1 mM for $[NaCl]_i = 0$, 25, 100 and 450 mM, respectively) is regarded as the critical micelle concentration for these systems.[2] It was found that $[Ca^{2+}]_f$ at $[DS^-]_f = 0$ mM increased with increasing $[NaCl]_i$. This increase can be explained in terms of ion exchange

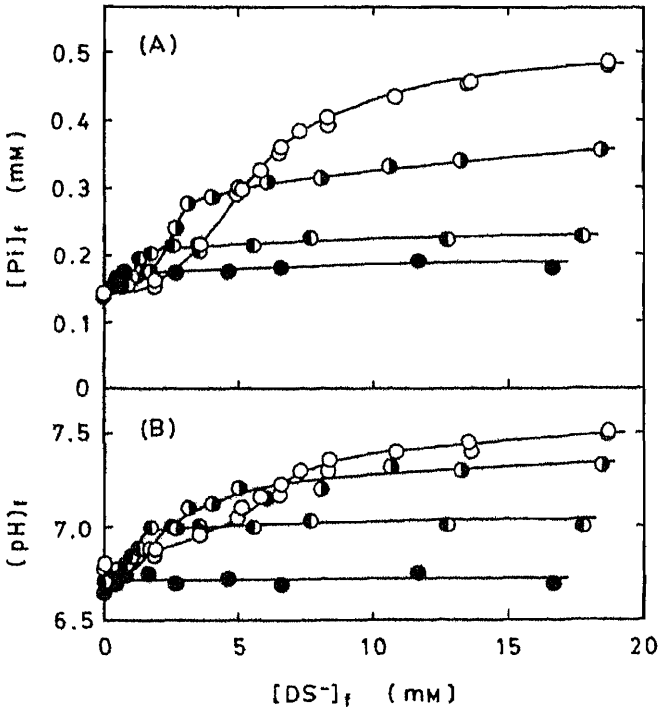

Fig. 3. (A) Relationship between Concentration of Free DS^- and That of Phosphate Ion Released from HAP at Constant Initial Concentration of Added NaCl

(B) Relationship between Concentration of Free DS^- and Equilibrium pH of the Solution at Constant Initial Concentration of Added NaCl

All the symbols are the same as those in Fig. 1.

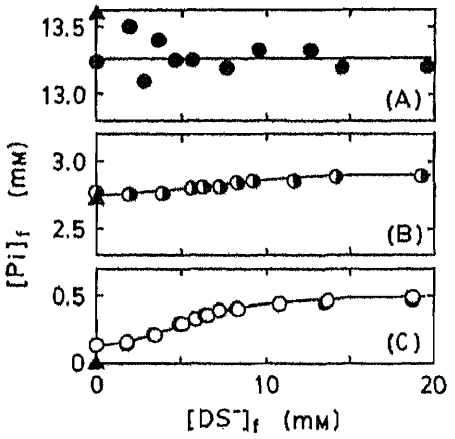

Fig. 4. Relationship between Concentration of Free DS^- and That of Phosphate Ion at Equilibrium at Constant Initial Concentration of Na_2HPO_4 Added

The equilibrium concentration of phosphate ion was lower or higher than the initial concentration of Na_2HPO_4 added (shown by the triangle on the ordinate), depending on the experimental conditions. Initial concentration of Na_2HPO_4 added, $[Na_2HPO_4]_i$ (mM): (A) 13.6, (B) 2.73, (C) 0.

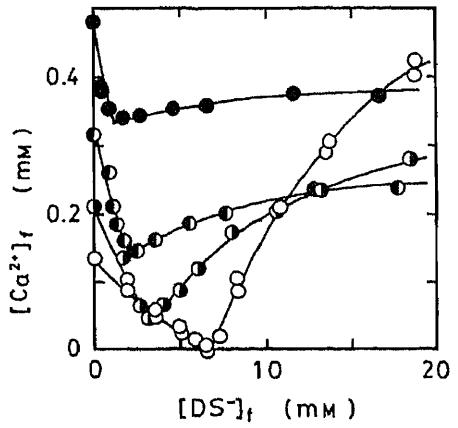

Fig. 5. Relationship between Concentration of Free DS^- and That of Calcium Ion Released from HAP at Constant Initial Concentration of Added NaCl

All the symbols are the same as those in Fig. 1.

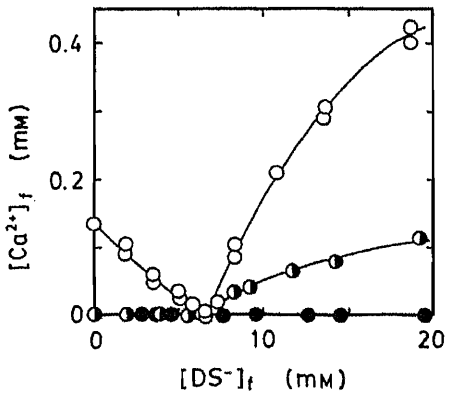

Fig. 6. Relationship between Concentration of Free DS^- and That of Calcium Ion Released from HAP at Constant Initial Concentration of Added Na_2HPO_4

All the symbols are the same as those in Fig. 2.

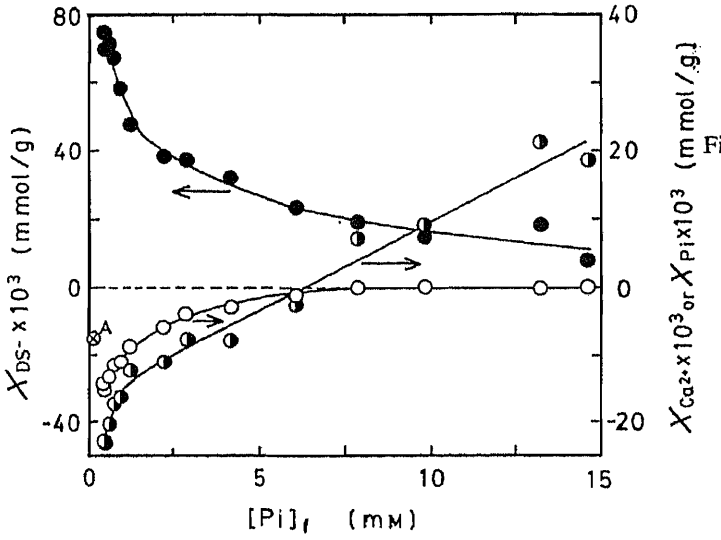

Fig. 7. Relationship between Equilibrium Concentration of Pi and the Adsorbed Amounts of DS^-, Pi and Ca^{2+}

○, $X_{Ca^{2+}}$; ◑, X_{Pi}; ●, X_{DS}. The initial concentration of SDS was 15 mM. All the equilibrium concentrations of DS^- for the data shown in this figure were higher than the cmc. The positive value in this figure (above the dotted line) shows the amount of adsorption, and the negative value (below the dotted line) gives the amount of ion released from HAP. The point A corresponds to the data on the ordinate in Fig. 4C, where neither Pi nor DS^- was added. The distance between point A and the curve for X_{Pi} (◑) indicates the effect of the presence of Pi and DS^-.

between Na^+ (of NaCl) and Ca^{2+} (on the surface of HAP) because substitution of Ca^{2+} and Na^+ occurs at the surface of HAP.[3] In addition, an increase in the ionic strength and concomitant decrease in the activity coefficients on the addition of NaCl may increase the analytical concentraion of Ca^{2+} at saturation.

Figure 6 shows the relationship between $[Ca^{2+}]_f$ and $[DS^-]_f$ in the presence of added Na_2HPO_4. It shows that when Na_2HPO_4 was added to the solution, $[Ca^{2+}]_f$ decreased with increasing $[Na_2HPO_4]_i$. The break points in Fig. 6 (corresponding to the critical micelle concentration (cmc))[2] are 6.7 and 6.3 mM for $[Na_2HPO_4]_i = 0$ and 2.73 mM, but no break point was found in the case of $[Na_2HPO_4]_i = 13.6$ mM.

Competitive Adsorption of DS^- and Pi

It is known that adsorption of phosphate ion on the HAP surface is specific[4] and stronger than those of other anions, such as chondroitin sulfate[5] and anionic salivary proteins.[6] Therefore, the effect of initial concentration of Pi on the amounts of adsorption and/or release of Pi, Ca^{2+} and DS^- was examined.

Figure 7 shows X_{DS^-}, X_{Pi} (the adsorbed amount of Pi), and $X_{Ca^{2+}}$ (the adsorbed amount

of Ca^{2+}) as a function of $[Pi]_f$, where Pi was supplied as Na_2HPO_4 and the initial concentration of SDS was kept constant at 15 mM. The value of $X_{Ca^{2+}}$ was negative because, as for Ca^{2+}, only release occurred. The value of X_{Pi} was also negative when $[Pi]_f$ was low. The results in Fig. 7 show that the release of Pi from HAP into the solution occurs during DS^- adsorption by the mechanism of ion-exchange between DS^- and Pi, as well as dissolution of HAP. The dissolved amount of Pi in the absence of both Na_2HPO_4 and SDS is shown by point A in Fig. 7 as a reference. The value of X_{Pi} increased with increasing $[Pi]_f$ and became positive when $[Pi]_f$ was higher than 6.2 mM. On the other hand, the released amount of Ca^{2+} decreased with increasing $[Pi]_f$, resulting in $X_{Ca^{2+}}=0$ around $[Pi]_f=7.8$ mM. The adsorbed amount of DS^- (X_{DS^-}) decreased monotonously with increase in X_{Pi}. The results show that competitive adsorption of DS^- and Pi takes place owing mainly to the mutual electrostatic repulsion between their negative charges.

Discussion

Effect of Added NaCl

Figure 8 shows the relationship between X_{DS^-} (taken from Fig. 1) and the increment of released amount of Pi due to the addition of SDS, $-\Delta X_{Pi}$, which was obtained from the difference of $[Pi]_f$ in the presence and absence of DS^- in the region of $[DS^-]_f<$cmc in Fig. 3A. The graph shows that $-\Delta X_{Pi}$ increases almost in proportion to X_{DS^-}. However, $-\Delta X_{Pi}$ decreases with increasing $[NaCl]_f$. This can be explained in terms of electrostatic shielding effect of NaCl added, because Na^+ concurrently binds to the adsorbed DS^- and to the surface of HAP as a counter ion, and, as a result, electrostatic repulsion between adsorbed DS^- and surface Pi is suppressed.

When $[DS^-]_f$ is lower than the cmc, $[Ca^{2+}]_f$ decreases, as shown in Fig. 5, with increasing $[Pi]_f$ to keep the solubility product for HAP, K_{sp}, constant.[2] The values of $-\log(Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2$, where ( ) means the activity for the ion in the parenthesis, were obtained in the range of $[DS^-]_f<$cmc according to the methods described previously.[2,7,8] The numerical values obtained were 113.1—117.6, 115.6—116.9, 117.9—119.3 and 127.6—128.4 for $[NaCl]_i=0$, 25, 100 and 450 mM, respectively. These numerical values were somewhat scattered, although the values of $-\log K_{sp}$ should be constant irrespective of the ionic strength. However, these are in the range of the literature values (=108—125)[9] except for the last ones (127.6—128.4). It is well-known that the Debye-Hückel approximation, which was used to obtain the activity coefficients in the present calculation, does not give the correct activity coefficient at high ionic strength. Therefore, the deviation of the calculated values from the literature values may be unavoidable since the ionic strength is as

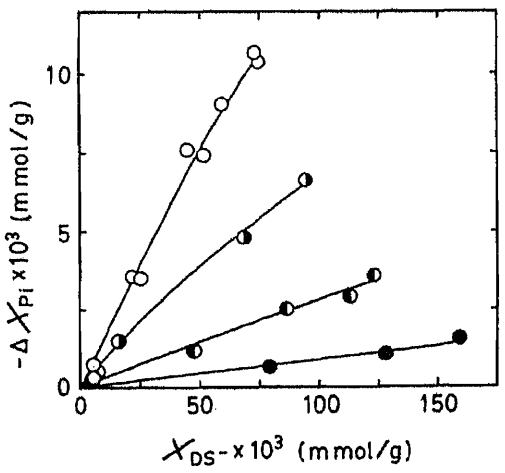

Fig. 8. Relationship between the Adsorbed Amount of DS^- and the Amount of Phosphate Ion Released through the Adsorption of DS^- at Constant Initial Concentration of Added NaCl

All the symbols are the same as those in Fig. 1. Concentrations of DS^- corresponding to X_{DS^-} (Fig. 1) and to $[Pi]_f$ (Fig. 3A) for this figure are less than the cmc (6.7, 3.2, 2.0 and 1.1 mM for $[NaCl]_i=0$, 25, 100 and 450 mM, respectively). The ordinate value, $-\Delta X_{Pi}$, was obtained from the data shown in Fig. 3A through the following equation, $-\Delta X_{Pi}=([Pi]_f$ in the presence of SDS$-[Pi]_f$ in the absence of SDS)/(weight of HAP added).

high as 450 mM NaCl.

On the other hand, in the region of $[DS^-]_f > cmc$, $[Ca^{2+}]_f$ increases with $[DS^-]_f$ by virtue of the binding of Ca^{2+} to DS^- micelles.[2] When NaCl was added to the solution, this increasing tendency of $[Ca^{2+}]_f$ (*i.e.*, positive slope of the curve) was suppressed as shown in Fig. 5. This can be explained in terms of the competitive binding of Ca^{2+} and Na^+ to DS^- micelles. That is, the ratio of the amount of Ca^{2+}-binding to that of Na^+-binding to the surface of micelles decreases when the concentration of added Na^+ increases. The negative charge remaining on the surface of HAP caused through Ca^{2+}-release from HAP to DS^- micelles is compensated by release of negative charges, *i.e.*, decreasing X_{DS^-} (Fig. 1) and releasing Pi (Fig. 3A). Accordingly, the negative slope of X_{DS^-} in Fig. 1 and the positive slope of $[Pi]_f$ in Fig. 3A above the cmc became less steep with increasing $[NaCl]_i$.

Effect of Added Na_2HPO_4

When $[DS^-]_f$ is lower than the cmc, $[Ca^{2+}]_f$ becomes too low to be detected in the presence of enough phosphate ion (Fig. 6). This may be explained as follows: adsorbed Pi prevents the release of Ca^{2+} from the surface of HAP owing to the electrostatic attractive force between them, and excess Pi in the solution suppresses Ca^{2+}-release because the concentraions of Pi and Ca^{2+} in the solution are mutually restricted so as to keep the solubility product for HAP constant.

Even in the region of $[DS^-]_f > cmc$, the Ca^{2+}-release from the surface of HAP is strongly depressed (Figs. 6 and 7). The competitive binding of Na^+ (from Na_2HPO_4) and Ca^{2+} (from HAP) to DS^- micelles takes part in this depression. Of course, two mechanisms, as mentioned above (adsorbed Pi and excess Pi in the solution), are also available. The latter makes the equilibrium concentration of Ca^{2+} free from both micelles and HAP low owing to the restriction of the constant solubility product of HAP.

Added Pi makes the adsorbed amount of DS^- (X_{DS^-}) decrease by competing for the adsorption sites on HAP, as shown in Fig. 7. On the other hand, Na^+ makes X_{DS^-} increase by reducing the electrostatic repulsion among dodecyl sulfate ions adsorbed on the surface of HAP by virtue of counter-ion binding and/or increasing the ionic strength. These opposite effects of Na^+ and Pi result in the complex behavior of the amount of adsorbed DS^-, as shown in Fig. 2.

In summary, the amount of DS^- adsorbed and the amounts of the lattice ions (Ca^{2+} and Pi) released synchronously from HAP depend on the species and concentration of added salt and on the concentration of DS^- micelles present in the solution.

The results mentioned above suggest that, in the human body, the affinity of organic ions for hard tissues is affected by the species and concentration of electrolytes present in the body fluids, and that some of the constituent ions of the hard tissues are released by the mechanisms of ion-exchange when organic ions are bound to the surface of hard tissues.

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On the Mechanism of Oxygenation of Olefins by Tetraphenylporphinatoiron(III)–Peroxide Systems[1)]

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The mode of oxygenation by *meso*-tetraphenylporphinatoiron(III)chloride (Fe(III)TPPCl)–peroxide systems was characterized by using *cis*-stilbene (**1**) and cholesteryl acetate (**3**) as substrates. The stereoselectivity of epoxidation of **1** by the Fe(III)TPPCl–*tert*-butyl hydroperoxide (^{t}BuOOH) system depended on the concentration of Fe(III)TPPCl; the reaction was nonstereoselective at 0.1 mM Fe(III)TPPCl but stereoselective at 10 mM Fe(III)TPPCl. In the reaction of **3** with the same system, both allylic oxidation and epoxidation proceeded. However, the epoxidation was β-selective independently of the concentration of Fe(III)TPPCl. Thus, ^{t}BuOOH is assumed to decompose by a radical chain reaction mechanism to give *tert*-butylperoxyl radical (^{t}BuOO·) and *tert*-butyloxyl radical (^{t}BuO·) at 0.1 mM Fe(III)TPPCl. At 10 mM Fe(III)TPPCl, on the other hand, the O–O bond in the Fe(III)TPPCl–^{t}BuOOH complex may cleave in both a homolytic manner (quasi-Fenton mechanism) and a heterolytic one (ferryl ion mechanism). The former leads to ^{t}BuO· as the active oxygen species responsible for the allylic oxidation of **3** and the latter to ferryl ion (Fe–O·) as that responsible for the stereoselective epoxidation of **1** and β-epoxidation of **3**.

In the reaction with the Fe(III)TPPCl–*m*-chloroperbenzoic acid (MCPBA) system at −30 °C in the presence of γ-collidine, β-selective epoxidation of **3** proceeded predominantly while **1** was epoxidized stereoselectively. The stereoselectivities of these epoxidations closely resemble those with the Fe(III)TPPCl–iodosylbenzene system, which is known to give the ferryl ion. These results suggest that the O–O bond in the Fe(III)TPPCl–MCPBA complex cleaves mainly in the heterolytic manner (ferryl ion mechanism).

Keywords——epoxidation stereoselectivity; cholesteryl acetate; *cis*-stilbene; *meso*-tetraphenylporphinatoiron(III)chloride; *tert*-butyl hydroperoxide; *m*-chloroperbenzoic acid, quasi-Fenton mechanism; oxyradical; ferryl ion

Cytochrome P-450 catalyzes oxygenations of a wide variety of organic compounds. The proposed mechanisms for the oxygenation involve the uptake of two electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH), the reduction of one atom of molecular oxygen to water and the insertion of the other into the substrate.[2)] The enzyme can also catalyze the hydroxylation of organic compounds without any electron donor or molecular oxygen if certain hydroperoxides (ROOH) are present (hydroperoxide-dependent hydroxylation).[2)] This hydroxylation has been assumed to proceed by the quasi-Fenton mechanism.[2)] Recently, several chemical models for the enzymatic hydroxylation have been studied by using ROOH and a metalloporphyrin such as *meso*-tetraphenylporphinatoiron(III)chloride (Fe(III)TPPCl).[3,4)] Groves *et al.* showed that the Fe(III)TPPCl–ROOH system oxidized cyclohexene by a free radical chain reaction mechanism.[3a)] On the other hand, several investigators have proposed that the quasi-Fenton mechanism operates in such reactions.[3d,4)]

The mode of oxygenation has often been investigated with *cis*-stilbene (**1**) as a typical substrate. The epoxidation of **1** by a nonradical reagent such as *m*-chloroperbenzoic acid

(MCPBA) is stereoselective, retaining the original geometry.[1b] In contrast, **1** is epoxidized nonstereoselectively by a free radical reagent such as peroxyl radical to give a mixture of *cis*- (**2a**) and *trans*- (**2b**) epoxides, since free rotation of the C–C bond is allowed in the formation of the radical intermediate.[1b] However, the epoxidation of **1** by the ferryl ion (Fe–O·), which has a radical character and is generated in the reaction of Fe(III)TPPCl and iodosylbenzene (PhIO), has recently been shown to be stereoselective owing to the participation of a solvent cage.[5] Thus, **1** cannot be used as a substrate for discrimination between oxygenations by a nonradical reagent and by the ferryl ion.

Our previous paper has demonstrated that the stereoselectivity in the epoxidation of cholesteryl acetate (**3**) also depends on the mode of oxygenation; nonradical reagents show α-selectivity, and radical ones including ferryl ions lead to β-selectivity, regardless of the participation of a solvent cage. Thus, by using both **1** and **3** as substrates, it would be possible to differentiate between oxygenations by nonradical reagents, free radical ones and the ferryl ion.[1b] In this paper, the mode of oxygenation by Fe(III)TPPCl–peroxide systems was characterized by examining the stereoselectivities in the epoxidations of **1** and **3**.

Results and Discussion

Epoxidations of *cis*-Stilbene (1) and Cholesteryl Acetate (3) by the Fe(III)TPPCl–*tert*-Butyl Hydroperoxide (^{t}BuOOH) System

The oxygenation of **1** and **3** by the Fe(III)TPPCl–^{t}BuOOH system was carried out in chlorobenzene at 25 °C for 1 h under an argon atmosphere. The oxygenations did not proceed in the absence of Fe(III)TPPCl. In the presence of catalytic amounts of Fe(III)TPPCl (0.1 mM), however, **1** was epoxidized nonstereoselectively by ^{t}BuOOH (200 mM) to give a mixture of **2a** and **2b** in a ratio of 2:98 (Table I). Under the same conditions, β-selective epoxidation and allylic oxidation took place in the oxygenation of **3** (Table II). These results are similar to those obtained in the oxygenations of **1** and **3** by the tris(acetylacetonato)iron(III) (Fe(acac)$_3$)–^{t}BuOOH system (Tables I and II). The Fe(acac)$_3$–^{t}BuOOH system has been shown to give *tert*-butyl peroxyl radical (^{t}BuOO·) and *tert*-butyloxyl radical (^{t}BuO·) through the Haber–Weiss mechanism,[6] and these radicals are responsible for the epoxidations of **1** and **3** and for the allylic oxidation of **3**, respectively.[7] Therefore, such radicals produced by a similar mechanism seem to participate in the oxygenations of **1** and **3** by the Fe(III)TPPCl (0.1 mM)-^{t}BuOOH system. However, the mode

Table I. Stereoselectivity of Epoxidation of *cis*-Stilbene

System	Epoxide	
	Yield (%)[a]	*cis*:*trans*
Fe(III)TPPCl (0.1 mM)-^{t}BuOOH[b]	3	2:98
Fe(III)TPPCl (10 mM)-^{t}BuOOH[b]	0.3	82:18
Fe(III)TPPCl (10 mM)-^{t}BuOOH-BHT[b,d]	0.03	88:12
Fe(acac)$_3$-^{t}BuOOH[b]	49	4:96
Fe(III)TPPCl-PhIO[b]	35	90:10
MCPBA[c]	0	
Fe(III)TPPCl (10 mM)-MCPBA[c]	2	83:17
MCPBA-collidine[c]	0	
Fe(III)TPPCl (10 mM)-MCPBA-collidine[c]	15	84:16
Fe(III)TPPCl (10 mM)-^{t}BuOOH-collidine[b]	0.6	83:17

a) Based on substrate. *b*) 25 °C, 1 h. *c*) −35 °C, 1 h. *d*) 60 mM BHT.

TABLE II. Oxygenations of Cholesteryl Acetate

System	Yield (%)[a]				Epoxidation (%)
	4 ($\beta/\alpha+\beta$)	5	6	7	
Fe(III)TPPCl (0.1 mM)–^{t}BuOOH[b]	0.1 (0.75)	0.6	0.3	0.1	9
Fe(III)TPPCl (10 mM)–^{t}BuOOH[b]	1 (0.75)	19	3	0.6	4
Fe(acac)$_3$–^{t}BuOOH[b]	1 (0.75)	77	3	11	11
Fe(III)TPPCl–PhIO[b]	19 (0.72)	0	5	0	79
MCPBA[c]	76 (0.33)	0	0	0	100
Fe(III)TPPCl (10 mM)–MCPBA[c]	26 (0.54)	0.2	2	0	92
MCPBA–collidine[c]	1	0	0	0	100
Fe(III)TPPCl (10 mM)–MCPBA–collidine[c]	25 (0.75)	0.5	4	0	85
Fe(III)TPPCl (10 mM)–^{t}BuOOH–collidine[b]	2 (0.75)	9	4	2	12

a) Based on substrate. *b*) 25 °C, 1 h. *c*) −35 °C, 1 h. *d*) 60 mM BHT.

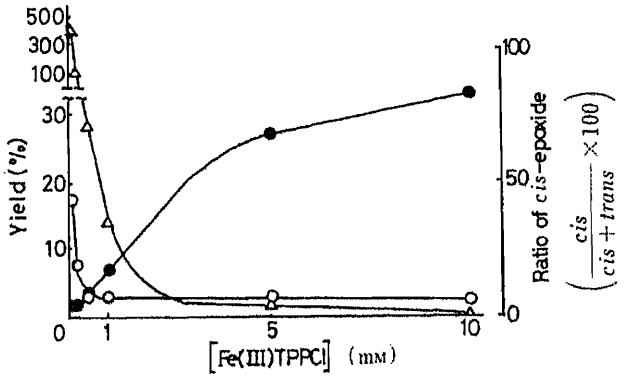

Fig. 1. Effect of the Concentration of Fe(III)-TPPCl on the Epoxidation of *cis*-Stilbene

The concentration of *tert*-butyl hydroperoxide was 200 mM. The yields of products were calculated on the basis of Fe(III)TPPCl. ○, the yield of *cis*-epoxide; △, the yield of *trans*-epoxide; ●, the ratio of *cis*-epoxide.

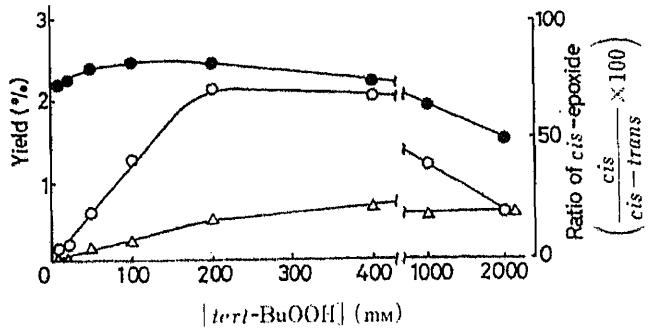

Fig. 2. Effect of the Concentration of ^{t}BuOOH on the Epoxidation of *cis*-Stilbene

The yields of products were calculated on the basis of Fe(III)TPPCl. The concentration of Fe(III)TPPCl was 10 mM. ○, the yield of *cis*-epoxide; △, the yield of *trans*-epoxide; ●, the ratio of *cis*-epoxide.

of the epoxidation of **1** by the Fe(III)TPPCl–^{t}BuOOH system was found to depend on the concentration of Fe(III)TPPCl; the stereoselectivity increased with increase in the concentration of Fe(III)TPPCl, though the total yield of the epoxides decreased, probably due to radical-induced decomposition of ^{t}BuOOH (Fig. 1).[8] As shown in Table I, the ratio of **2a** to **2b** reached 82 : 18 at 10 mM Fe(III)TPPCl. The effects of ^{t}BuOOH concentraion on the mode of the reaction are shown in Fig. 2. The yield of epoxides increased with increase in the concentration of ^{t}BuOOH up to 200 mM and remained constant over the range of 200—400 mM. The ratio of **2a** and **2b** remained nearly unchanged over the range of 10—400 mM ^{t}BuOOH, indicating that the mode of the epoxidation is not affected by the concentration of ^{t}BuOOH. The oxygenation of **3** by the Fe(III)TPPCl–^{t}BuOOH system was also carried out at 10 mM Fe(III)TPPCl, and the results were compared with those at 0.1 mM Fe(III)TPPCl

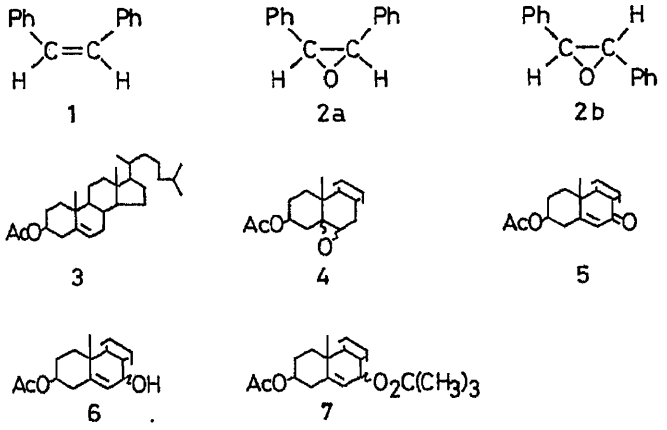

Chart 1

(Table II). Though the yields of α-epoxide (**4a**), β-epoxide (**4b**) and the allylic oxidation products (**5**, **6**, and **7**) were higher at 10 mM than at 0.1 mM Fe(III)TPPCl, stereoselectivity in the epoxidation was independent of the concentration of Fe(III)TPPCl. Therefore, the main epoxidizing agent produced in the Fe(III)TPPCl–^{t}BuOOH system at 10 mM Fe(III)TPPCl is concluded to be neither nonradical nor radical (other than the ferryl ion), since nonradical and radical reagents catalyze α-selective epoxidation of **3** and nonstereoselective epoxidation of **1**, respectively.[1b)]

Possible reaction mechanisms between Fe(III)TPPCl and ^{t}BuOOH are assumed to be: (i) bonding of ROOH to Fe(III)TPPCl, followed by homolytic cleavage of the O–O bond in the complex to give an alkoxyl radical and hydroxo ferryl ion complex (quasi-Fenton mechanism), (ii) heterolytic cleavage of the O–O bond in the complex to afford the ferryl ion (ferryl ion mechanism) (Chart 2). An alkoxyl radical is unable to epoxidize an olefin, though the radical can abstract a hydrogen atom from a hydrocarbon. The hydroxo ferryl ion (Fe(IV)–OH) may not oxidize an organic compound. On the other hand, the ferryl ion (Fe(IV)–O·) which is formed in the reaction between Fe(III)TPPCl and PhIO, is capable of epoxidizing an olefin as well as abstracting a hydrogen atom from a hydrocarbon. Since the stereoselectivities in the epoxidations of **1** and **3** by the Fe(III)TPPCl–^{t}BuOOH system were similar to those by the Fe(III)TPPCl–PhIO system (Tables I and II), the ferryl ion produced by heterolytic cleavage may participate as a main epoxidizing agent in the oxygenations of **1** and **3** by the Fe(III)TPPCl (10 mM)–^{t}BuOOH system.

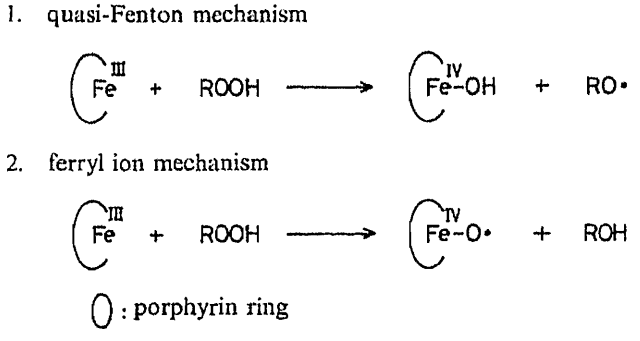

Chart 2

Though the ferryl ion may also participate in the allylic oxidation of **3** with Fe(III)TPPCl–^{t}BuOOH, the extent of the allylic oxidation was greater than that with the Fe(III)TPPCl–PhIO system. Further, the ratio of epoxides to the allylic oxidation products

from the reaction with the Fe(III)TPPCl–^{t}BuOOH system was similar not to those with the Fe(III)TPPCl–PhIO system but to those with the Fe(acac)$_3$–^{t}BuOOH system. Thus, in competition with the heterolytic cleavage of the O–O bond in the complex of Fe(III)TPPCl–^{t}BuOOH (ferryl ion mechanism in Chart 2), the homolytic cleavage (quasi-Fenton mechanism) seems to occur to give ^{t}BuO· (responsible for the allylic oxidation of **3**). An alkoxyl radical can induce the decomposition of ROOH to give the alkylperoxy radical, leading to nonstereoselective epoxidation of **1** and β-selective epoxidation of **3**. In the oxygenation by the Fe(III)TPPCl–^{t}BuOOH system, such radical-induced decomposition may occur to give ^{t}BuOO·, which may participate partially in the nonstereoselective epoxidations of **1** and β-selective epoxidation of **3**. In fact, the stereoselectivity in the epoxidation of **1** by the Fe(III)TPPCl–^{t}BuOOH system increased in the presence of a radical scavenger, di-*tert*-butylhydroxytoluene (BHT), and closely resembled that obtained with the Fe(III)TPPCl–PhIO system (Table I). It is, therefore, assumed that the Fe(III)TPPCl–^{t}BuOOH system produces the ferryl ion and ^{t}BuO· as active oxidants through heterolytic and homolytic cleavages of the O–O bond in the complex of Fe(III)TPPCl and ^{t}BuOOH, respectively.

Epoxidation of 1 and 3 by the Fe(III)TPPCl–MCPBA System

Recently, the mode of cleavage of the O–O bond in metalloporphyrin–ROOH complexes has been discussed on the basis of kinetic analysis of the reactions between metalloporphyrins and a series of ROOH including percarboxylic acids (R=acyl).[4] These kinetic studies suggested that the mode of cleavage depends on the structure of the peroxide; the ease of heterolytic cleavage increases as R in ROOH becomes more electron-withdrawing. Thus, the oxygenations of **1** and **3** were carried out with the Fe(III)TPPCl–MCPBA system, in which the heterolytic cleavage seems to be favorable. The results obtained are summarized in Tables I and II. Since **1** and **3** are epoxidized by MCPBA alone at 25 °C *via* a nonradical process,[9] inhibition of these epoxidations is necessary to characterize the mode of oxygenation by the Fe(III)TPPCl–MCPBA system. Though MCPBA did not epoxidize **1** at −30 °C, the epoxidation of **3** proceeded even at −30 °C. The epoxidations of both **1** and **3** were inhibited almost completely by addition of γ-collidine (2,4,6-trimethylpyridine). Collidine can act as a base, but cannot cordinate to iron porphyrin because of its steric hindrance.[10] Thus, collidine seems not to have any effect on the mode of the oxygenation by the Fe(III)TPPCl–MCPBA system. In fact, the presence of γ-collidine did not alter the mode of oxygenation of **1** and **3** by the Fe(III)TPPCl–^{t}BuOOH system. In the oxygenations of both **1** and **3** by the Fe(III)TPPCl–MCPBA system at −30 °C in the presence of γ-collidine, the extents of epoxidation increased significantly as compared with the results obtained with the Fe(III)TPPCl–^{t}BuOOH system. Since the yields of epoxides in both reactions exceeded 100% on the basis of Fe(III)TPPCl, the iron played a catalytic role. Furthermore, the product distributions and stereoselectivities in the epoxidations closely resembled those with the Fe(III)TPPCl–PhIO system. Therefore, the Fe(III)TPPCl–MCPBA system may give Fe–O· as a main active oxygen through the heterolytic cleavage of the O–O bond in the Fe(III)TPPCl–MCPBA complex. This conclusion is in agreement with those of Lee and Bruice[4] and Traylor *et al.*[10]

From the results obtained in this study, it may be concluded that the mode of oxygenation by the Fe(III)TPPCl–peroxide system depends on the concentration of Fe(III)TPPCl as well as the structure of the peroxide. In the presence of catalytic amounts of Fe(III)TPPCl (0.1 mM), ^{t}BuOOH decomposes *via* a free radical chain reaction to give ^{t}BuO· and ^{t}BuOO·, which are responsible for allylic oxidation and epoxidation, respectively. At a high concentration (10 mM of Fe(III)TPPCl), however, the O–O bond in the Fe(III)TPPCl and ^{t}BuOOH complex cleaves both heterolytically and homolytically. The former leads to Fe–O· and the latter to ^{t}BuO· as active oxygen species. On the other hand, when MCPBA is used instead of ^{t}BuOOH, Fe–O· is formed as a main active oxygen species through the

heterolytic cleavage of the O–O bond in the Fe(III)TPPCl–MCPBA complex.

The mechanism of camphor hydroxylation by the lipoate–cytochrome P-450 system has been reported to involve acylation of heme-bound dioxygen by the carboxylate of lipoate.[11] It is of interest that oxygenation by the Fe(III)TPPCl–MCPBA–collidine system, a chemical model for the enzymatic reaction, proceeds with the ferryl ion (Fe–O·) as an attacking species. Our results suggest that P-450-catalyzed oxygenation involves both heterolytic and/or homolytic cleavage of the O–O bond in the oxidant, depending on its structure.

Experimental

General Methods——Gas chromatograms were taken on a Shimadzu GC-4CM machine. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a JEOL JNM-FX 100FT spectrometer at 100 MHz with tetramethylsilane as an internal standard in $CDCl_3$. Determinations of the yield and the epimeric ratio of **2a** and **2b** were carried out by the reported gas liquid chromatography (GLC) method (column, 2% OV 225 (2 m × 3 mm i.d.); column temp., 150 °C).[1b] The epimeric ratio of **4a** and **4b** was determined by the ^{1}H-NMR method previously reported.[12] Radioactivity was measured with a Packard Tri-Carb 460C. For thin-layer chromatography (TLC), silica gel (Wakogel B-5F) was used as an adsorbent.

Materials——Fe(III)TPPCl was prepared by the method of Adler *et al.*[13] Cholesteryl acetate (**3**) and authentic samples (**4**—**7**) were prepared by the methods reported previously.[14] [4-^{14}C]Cholesteryl acetate (5.0×10^4 cpm/mg) was prepared from [4-^{14}C]cholesterol (60.0 mCi/mmol, from Japan Radioisotope Association) by the cited method.[15] ^{t}BuOOH (Nakarai Chem. Co.) was purified by distillation under reduced pressure. The purity was determined to be 94% by iodometry. Other reagents were purchased from commercial sources and were used without purification.

Oxygenation of 1 in the Fe(III)TPPCl–^{t}BuOOH System——A chlorobenzene solution (1.0 ml) of **1** (0.12 mmol), Fe(III)TPPCl (0.1 or 10 μmol) and ^{t}BuOOH (0.20 mmol) was stirred for 1 h at 25 °C under an argon atmosphere. A portion (0.1 ml) of the reaction mixture was subjected to chromatography on silica gel (1 g) and eluted with benzene (10 ml). The benzene eluate was evaporated to dryness. The residue was redissolved in 0.1 ml of acetone containing benzophenone as an internal standard. The solution was subjected to GLC for determination of the yield and the isomer ratio of the resulting epoxides. The effect of BHT or γ-collidine was tested by addition of 0.06 mmol of BHT or 0.23 mmol of γ-collidine. Under the same conditions, the effect of various concentrations of Fe(III)TPPCl (0.1—10 mM) or ^{t}BuOOH (10—2000 mM) on this reaction was examined at 200 mM ^{t}BuOOH or 10 mM Fe(III)TPPCl, respectively.

Oxygenation of 3 in the Fe(III)TPPCl–^{t}BuOOH System——A chlorobenzene solution (1.0 ml) of **3** (4-^{14}C labeled, 0.12 mmol), Fe(III)TPPCl (0.1 or 10 μmol) and ^{t}BuOOH (0.20 mmol) was stirred for 1 h at 25 °C under an argon atmosphere. A portion (20 μl) of the reaction mixture was diluted with benzene containing carrier steroids (**3**—**7**), subjected to TLC and developed with benzene–ethyl acetate (19 : 1, for **4**, **5**, and **6**) or *n*-hexane–benzene (6 : 4, ×3, for **3** and **7**). The steroids on the TLC plates were visualized with iodine vapor. After the iodine had evaporated, the corresponding zones were scraped into counting vials. The radioactivities were measured for the determination of the product distribution. For the determination of the epimeric ratio of the epoxides (**4a** and **4b**), an unlabeled substrate was used for the oxygenation. The epoxides were separated from the reaction mixture in a similar manner, and the epimeric ratio was determined by the ^{1}H-NMR method.[12]

Epoxidation of 1 or 3 by MCPBA——MCPBA (0.10 mmol) was added to a chlorobenzene solution (1.0 ml) of **1** or **3** (0.12 mmol), and the mixture was allowed to stand at −30 °C for 1 h under an argon atmosphere. The reaction mixture was worked up, and the product distribution and the epimeric ratio of the epoxides were determined. Under the same conditions, the oxygenation of **1** or **3** with MCPBA was carried out in the presence of γ-collidine (0.23 mmol).

Oxygenation of 1 or 3 in the Fe(III)TPPCl–MCPBA System in the Presence of γ-Collidine——MCPBA (0.10 mmol) was added to a chlorobenzene solution (1.0 ml) of **1** or **3** (0.12 mmol), Fe(III)TPPCl (0.01 mmol) and γ-collidine (0.23 mmol). The mixture was allowed to stand at −30 °C for 1 h under an argon atmosphere, then treated as described above for the determination of products and the epimeric ratio of the epoxides.

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References and Notes

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Highly Stereoselective Total Synthesis of Methynolide, the Aglycon of the 12-Membered Macrolide Antibiotic Methymycin. I. Synthesis of a Prelog–Djerassi Lactone-Type Chiral Intermediate from D-Glucose[1,2]

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For the highly stereoselective synthesis of methynolide (**2**), the aglycon of the 12-membered macrolide antibiotic methymycin (**1**), a Prelog–Djerassi lactone-type chiral intermediate (**7a**) bearing four chiral centers corresponding to the C-2, C-3, C-4, and C-6 positions was synthesized from D-glucose. In this synthesis, several stereocontrolled reactions such as hydroboration, catalytic hydrogenation, *etc.* were successfully applied. The utility of the 4-methoxybenzyl protecting group was also demonstrated.

Keywords——macrolide antibiotic; aglycon; methynolide; acyclic stereocontrol; hydroboration; catalytic hydrogenation; protecting group; stereoselective synthesis

Macrolide antibiotics with multiple chiral centers, owing to the presence of many substituents and functional groups, have received much recent synthetic attention because of their significant pharmacological and biological activities.[3] For the total synthesis of such complex compounds, new synthetic methodologies mainly consisting of means of stereochemical control in acyclic systems, selective use of suitable protecting groups, and efficient macro-cyclizations are primarily required.

As part of the synthetic effort directed towards polyketide-derived natural products, macrolide and polyether antibiotics, our attention has recently been focused on the chiral synthesis of some representative antibiotics, methymycin (**1**),[4] pikromycin,[5] erythromycin A,[6] tylosine,[7] iso-lasalocid A,[8] salinomycin,[9] *etc.*, from D-glucose as a chiral starting material.

In order to establish our synthetic methodology, which is widely applicable to the synthesis of complex antibiotics, and mainly consists of some acyclic stereocontrolled reactions and the use of benzyl-type protecting groups,[10] we first planned a highly stereoselective synthesis of methynolide (**2**),[11] the aglycon of the 12-membered macrolide antibiotic methymycin (**1**). Our retrosynthesis of **2**, consisting of two routes, route a *via* the lactonization of the known seco-acid (**3**) and route b *via* the Wittig–Horner reaction of **4**, is shown in Chart 1. Both segment i and segment ii for the synthesis of **3** or **4** were expected to be synthesized from a ulose (**5**). In the present paper, we report stereoselective syntheses of a Prelog–Djerassi lactone-type chiral intermediate (**7a**) from D-glucose *via* **5**.

Results and Discussion

The Prelog–Djerassi lactonic acid (**8**),[12] corresponding to the C-1—C-7 segment of **2**, has

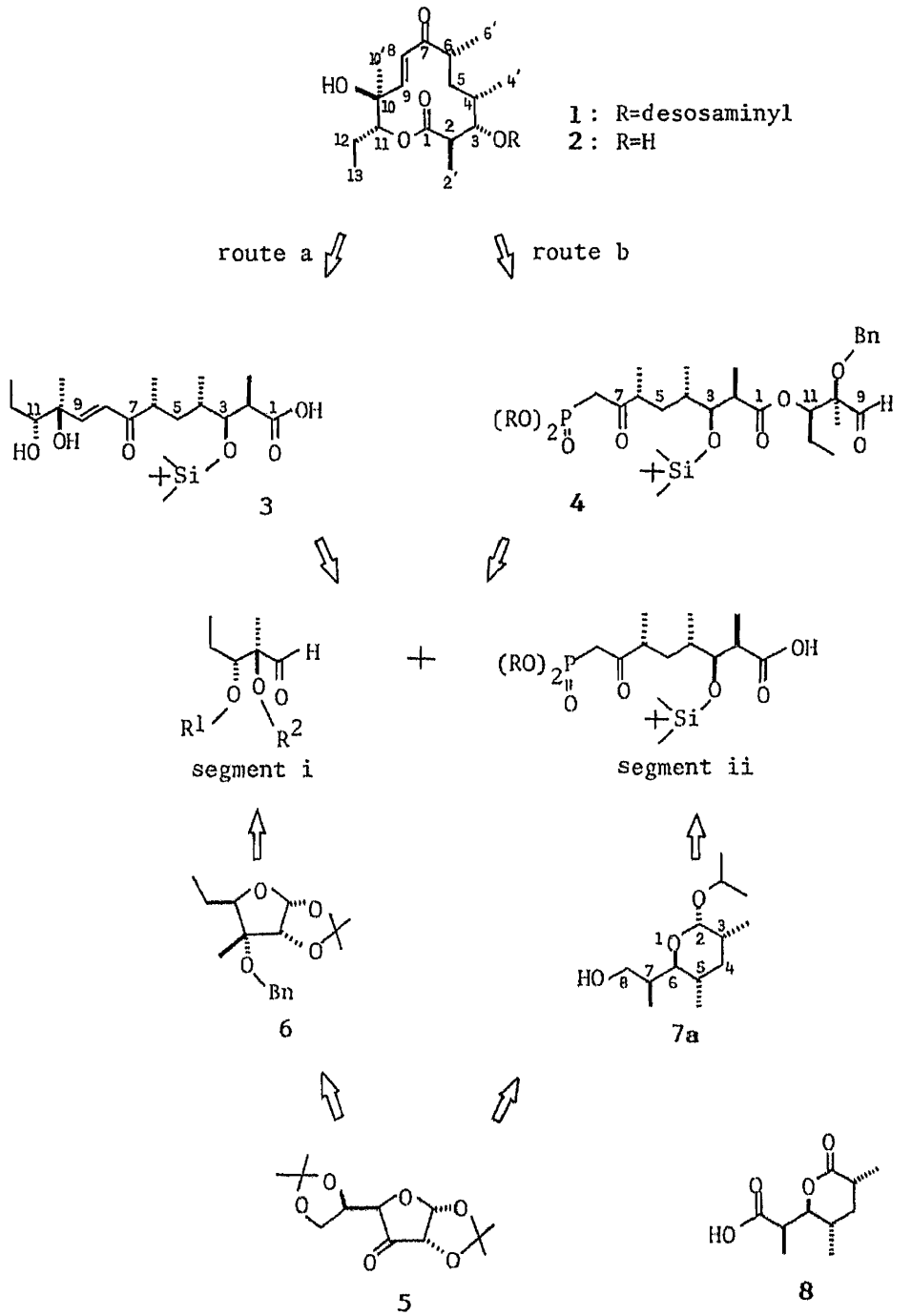
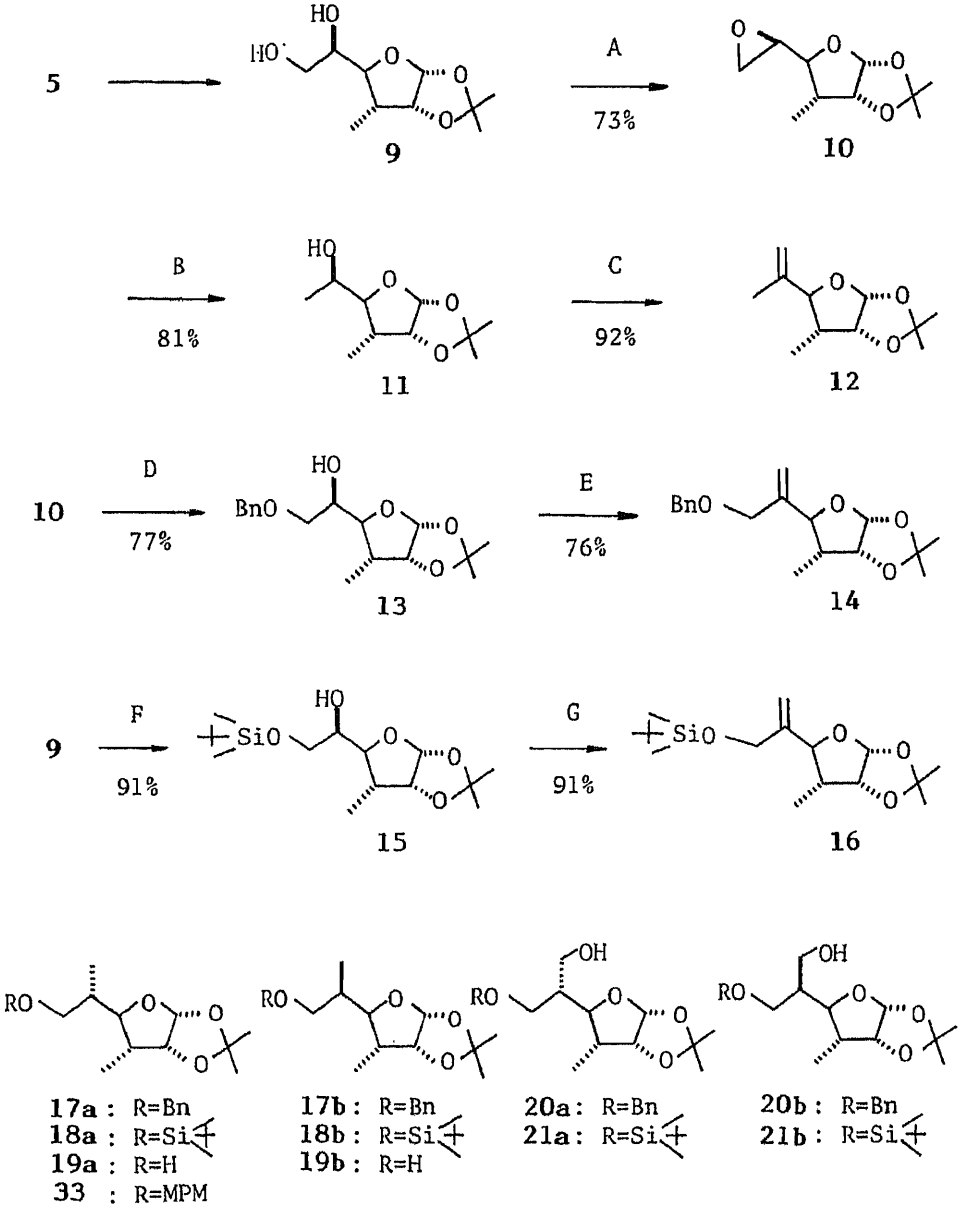


Chart 1

been a key compound for both the structure elucidation of **1** and subsequent synthetic efforts. The title compound (**7a**) is considered to be a more versatile synthetic equivalent of **8**.

For the synthesis of **7a**, we first synthesized the key intermediate (**19a**), with three contiguous chiral centers corresponding to C-2—C-4 of **2**, from D-glucose *via* catalytic reduction or hydroboration of **12**, **14**, or **16** with acyclic stereocontrol (Chart 2). The known diol (**9**),[13] derived from **5**, was converted to the epoxide (**10**) *via* a monotosylate. Reduction with lithium aluminium hydride (LAH) of **10** gave the secondary alcohol (**11**), which was oxidized with pyridinium chlorochromate (PCC) followed by the usual Wittig methylenation to give the olefin (**12**). On the other hand, nucleophilic ring opening of **10** with sodium



(A) 1) TsCl, pyridine; 2) K_2CO_3, MeOH (B) LAH, Et_2O (C) 1) PCC, molecular sieves, CH_2Cl_2; 2) $Ph_3P{=}CH_2$, THF (D) BnONa, DMSO–THF (E) 1) PCC, molecular sieves, CH_2Cl_2; 2) $Ph_3P{=}CH_2$, THF (F) TBDMSCl, imidazole, CH_2Cl_2 (G) 1) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, $-50\,°C$; 2) $Ph_3P^+Me\cdot Br^-$, *n*-BuLi, THF, 0 °C

Chart 2

benzyloxide gave the hydroxybenzyl ether (**13**), which was similarly converted to the second olefin (**14**) in reasonable yield. The third olefin (**16**) was also easily synthesized from **9** *via* three conventional reactions, selective *tert*-butyldimethylsilyl (TBDMS) protection of the primary alcohol, Swern oxidation of the secondary alcohol and the final Wittig methylenation.

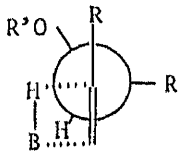
Hydrogenation of **14** over 10% palladium on charcoal (Pd–C) in ethyl acetate (EtOAc) gave a stereoisomeric mixture (1.3 : 1) with a slight excess of the desired isomer (**17a**). The ratio of **17a** and **17b** was improved to 4 : 1 by the use of 5% rhodium on alumina ($Rh–Al_2O_3$) catalyst, though this is still unsatisfactory (Table I). Almost no selectivity was observed in the hydrogenation of **16** over both Pd–C and $Rh–Al_2O_3$ catalysts; a *ca.* 1 : 1 mixture of **18a** and

TABLE I. Catalytic Hydrogenation and Hydroboration Results

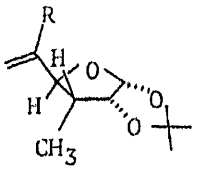
Substrate	Conditions	Product	
		Yield (%)	Ratio
14	10% Pd–C, H_2, EtOAc	**17a, b** (85)	1.3:1
14	Rh–Al_2O_3, H_2, EtOH	**17a, b** (100)	4:1
12	BH_3, THF	**19a, b** (59)	1:6.8
14	BH_3, THF	**20a, b** (82)	24:1
16	BH_3, THF	**21a, b** (91)	11:1
22	BH_3, THF	**25a, b** (65)	1:6.0
23	BH_3, THF	**26a, b** (75)	1:1.8
24	BH_3, THF	**27a, b** (69)	1:4

18b was obtained. Therefore, we turned our attention to hydroboration.

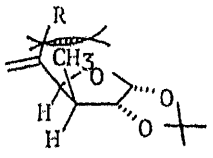
When **12** was treated with diborane, smooth hydroboration occurred to give a 6.8:1 mixture of **19b** and **19a**. Unfortunately, the major product was the undesired form. Recently, Still and Barrish[14] and Houk *et al.*[15] discussed transition state structures in the hydroboration of asymmetric allyl alcohol derivatives, and concluded that the conformation with the C–O bond *anti* to the double bond was most favorable, as shown in Chart 3. The result of hydroboration of **12** is consistent with their discussion, namely the *si*-face attack in the M-1 structure (Chrat 3) with diborane was predominant. For the same reason, better stereoselectivities (*re*-face attack of diborane) were obtained in the hydroboration of **14** and **16** to give mainly the desired products, **20a** (96:4) and **21a** (92:8), respectively (Table I).[16] Compounds **20a** and **21a**, without purification, were readily converted to the key intermediate (**19a**) by means of conventional reactions, tosylation, LAH reduction, *etc*. Benzyl protection of the primary alcohol of **19a** readily gave **17a**.



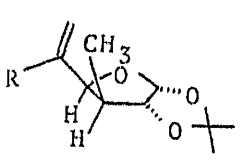
Still's model



M-1



M-2



M-3

12 : R= CH_3
14 : R= CH_2OBn
16 : R= $CH_2OSi\leqslant$

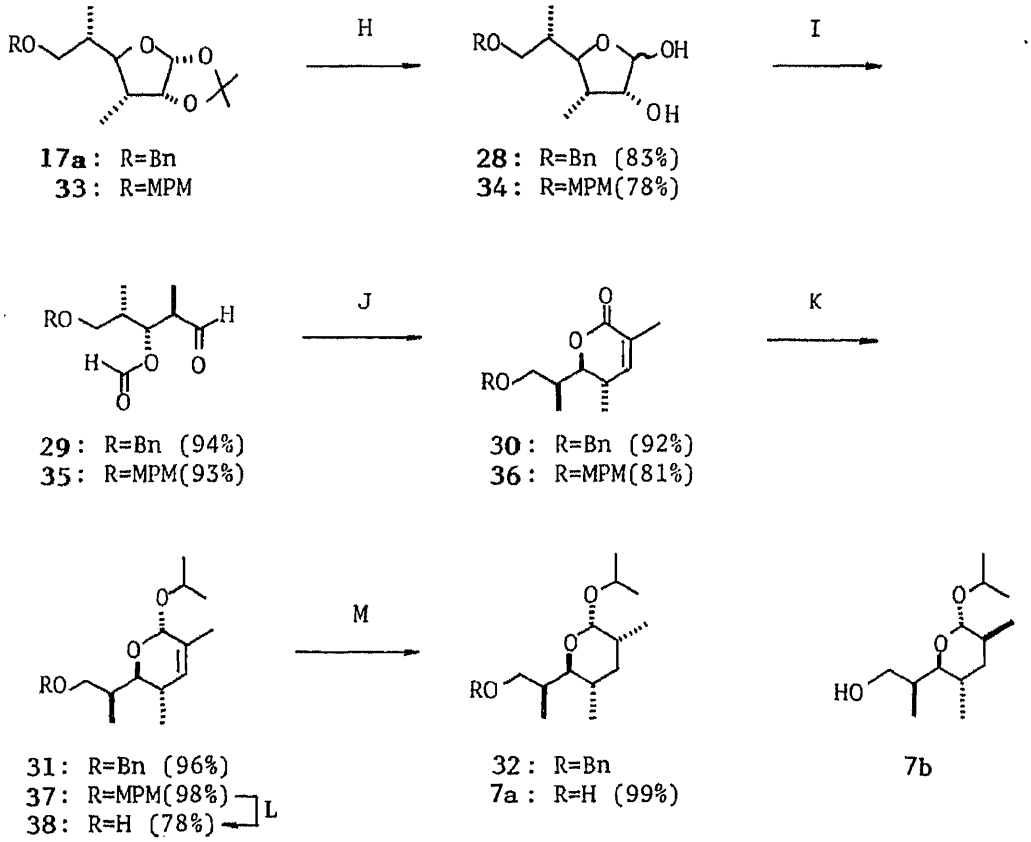
Chart 3

The three contiguous chiral centers corresponding to C-5—C-7 of the title compound (**7a**) [*i.e.*, to C-2—C-4 of **2**] were thus constructed, and the final chiral center at C-3 [C-6 of **2**] was introduced stereoselectively by hydrogenation of α-lactolide derivatives as follows (Chart 4).

The acetonide protection of **17a** was removed with hydrochloric acid and the resultant lactol (**28**) was oxidized with sodium periodate to give the aldehyde (**29**). When **29** was treated with the sodium salt of dimethyl 1-methoxycarbonylethylphosphonate at −90 °C, the Wittig–Horner reaction[17] proceeded quite smoothly to give the (*Z*)-α,β-unsaturated ester (9.6:1

stereoselectivity), which, without purification, was converted to the α,β-unsaturated lactone (**30**) by treatment with methanolic potassium carbonate. Catalytic reduction of the double bond of **30** was expected to give a so-called Prelog–Djerassi lactone-type compound, but in order to increase the stereoselectivity of the reduction, **30** was converted to the anomerically pure α-lactolide (**31**) *via* reduction with diisobutylaluminum hydride (DIBAH) followed by isopropyl protection of the resultant lactol.[18]

When **31** was hydrogenated over 10% Pd–C in EtOAc at 0 °C, **32** was obtained in high yield, though the stereoselectivity (6 : 1) was still unsatisfactory. Debenzylation of **32**, without purification, over Raney nickel (Ni) W-2[19] readily gave the title compound (**7a**) after chromatographic purification. When **31** was reduced over Rh–Al_2O_3 in ether, concomitant saturation of the benzyl group was unavoidable. After several examinations of substrates and catalysts in order to increase the stereoselectivity, an excellent result was obtained in the reduction of **38**, which was synthesized from the key intermediate (**19a**) *via* **33**—**37** by a method virtually identical with that described for **31**, forllowed by removal of the 4-methoxybenzyl (MPM) protection of **37** with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). The deprotection was smoothly carried out by a slight modification of the usual method,[10] namely in the presence of isopropanol, in good yield.[19] Hydrogenation of **38** over Rh–Al_2O_3 in ether gave the desired **7a** with excellent stereoselectivity (25 : 1) in quantitative yield. Compound **7a** has all the chiral centers required for segment ii, whose synthesis will be



(H) 4N HCl, THF or dioxane, 40—45 °C (I) $NaIO_4$, MeOH–H_2O, 0 °C or $Pb(OAc)_4$, benzene (J) 1) $(MeO)_2POCH(Me)CO_2Me$, NaH, THF, −80→8 °C; 2) K_2CO_3, MeOH (K) 1) DIBAH, toluene, −80 °C; 2) CSA, iso-PrOH (L) DDQ, CH_2Cl_2–H_2O–iso-PrOH (M) a: **31**→**7a**; 1) Pd–C, H_2, EtOAc; 2) Raney Ni (W-2), H_2, EtOH b: **38**→**7a**; Rh–Al_2O_3, H_2, Et_2O

Chart 4

described in the following paper.

Experimental

Unless otherwise noted, physical data were measured as follows. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-200 (200 MHz) or JEOL JNM GX-270 (270 MHz) instrument. Mass spectra (MS) were taken on a JEOL JMS D-300 or JEOL JMS-01 SG spectrometer. Infrared (IR) spectra were recorded on a JASCO IR-2 spectrometer.

5,6-Anhydro-3-deoxy-1,2-*O*-isopropylidene-3-*C*-methyl-α-D-allofuranose (10)——*p*-Toluenesulfonyl chloride (TsCl) (4.91 g, 25.7 mmol) was added portionwise to a stirred solution of **9**[13] (5.61 g, 25.7 mmol) in pyridine (36 ml) over a period of 7 h. After an additional 17 h, the reaction mixture was poured into ice-water (150 ml) and extracted with CH_2Cl_2. The extract was washed with 2N HCl, brine and 5% $NaHCO_3$, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was dissolved in MeOH (120 ml), and treated with K_2CO_3 (4 g) at room temperature for 40 min. The reaction mixture was evaporated *in vacuo*, and the residue was extracted with CH_2Cl_2. The extract was washed with brine, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (4:1) to afford **10** as a colorless oil (3.78 g, 73.4%). ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, d, J=7 Hz), 1.33 (3H, s), 1.50 (3H, s), 2.03 (1H, ddq, J=4.5, 10, 7 Hz), 2.68 (1H, dd, J=2.5, 5 Hz), 2.78 (1H, dd, J=4, 5 Hz), 2.92 (1H, ddd, J=2.5, 4, 6 Hz), 3.51 (1H, dd, J=6, 10 Hz), 4.57 (1H, dd, J=3.5, 4.5 Hz), 5.81 (1H, d, J=3.5 Hz). MS *m/z* (relative intensity): 185 (M^+-15, 50), 157 (10), 99 (20), 59 (56), 43 (100). Exact MS *m/z* Calcd for $C_9H_{13}O_4$ (M^+-15): 185.0814. Found: 185.0811.

3,6-Dideoxy-1,2-*O*-isopropylidene-3-*C*-methyl-α-D-allofuranose (11)——A solution of **10** (6.78 g, 33.9 mmol) in Et_2O (17 ml) was added dropwise to a stirred ice-cold suspension of LAH (0.845 g, 22.2 mmol) in Et_2O (63 ml). The mixture was stirred for 5 h at 0 °C and then 1 h at room temperature. Usual work-up gave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (3:2) to afford **11** as a colorless oil (5.55 g, 81%). ^{1}H-NMR ($CDCl_3$) δ: 1.14 (3H, d, J=7 Hz), 1.21 (3H, d, J=7 Hz), 1.34 (3H, s), 1.52 (3H, s), 1.99 (1H, d, J=4 Hz), 2.10 (1H, ddq, J=5, 10, 7 Hz), 3.80 (1H, dd, J=3, 10 Hz), 4.20 (1H, ddq, J=3, 4, 7 Hz), 4.56 (1H, dd, J=4, 5 Hz), 5.77 (1H, d, J=4 Hz). MS *m/z* (relative intensity): 187 (M^+-15, 30), 157 (48), 99 (77), 71 (53), 59 (93), 43 (100). Exact MS *m/z* Calcd for $C_9H_{15}O_4$ (M^+-15): 187.0970. Found: 187.0982.

3,5,6-Trideoxy-1,2-*O*-isopropylidene-3-*C*-methyl-5-methylene-α-D-*ribo*-hexofuranose (12)——A solution of **11** (0.554 g, 2.52 mmol) and PCC (1.20 g, 5.57 mmol) in CH_2Cl_2 (35 ml) with powdered 3 Å molecular sieves (4.2 g) was stirred for 6 h at room temperature. After removal of insoluble materials by filtration, the filtrate was evaporated *in vacuo* and the residue was extracted with ether. The extract was purified by passage through a silica gel column with ether to give the ketone as a colorless oil (0.463 g, 92%). IR ν_{max} cm^{-1}: 1720. ^{1}H-NMR ($CDCl_3$) δ: 1.16 (3H, d, J=7 Hz), 1.35 (3H, s), 1.51 (3H, s), 2.00 (1H, ddq, J=5, 11, 7 Hz), 4.08 (1H, d, J=11 Hz), 4.58 (1H, dd, J=3, 5 Hz), 5.91 (1H, d, J=3 Hz). MS *m/z* (relative intensity): 185 (M^+-15, 12), 157 (55), 99 (58), 85 (18), 71 (50), 59 (80), 43 (100). Exact MS *m/z* Calcd for $C_9H_{13}O_4$ (M^+-15): 185.0814. Found: 185.0812.

A tetrahydrofuran (THF) solution (1 ml) of the above ketone (0.290 g, 1.45 mmol) was added dropwise to a stirred solution of methylenetriphenylphosphorane, prepared from NaH (0.104 g, 4.33 mmol) in dimethylsulfoxide (DMSO) and methyltriphenylphosphonium bromide (1.65 g, 4.62 mmol), at 18 °C. After 2 h, the reaction mixture was poured into ice-cold saturated NH_4Cl and extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (3:2) as the eluent to afford **12** as a colorless oil (0.287 g, 100%). ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, d, J=7 Hz), 1.34 (3H, s), 1.53 (3H, s), 1.71 (3H, s), 1.88 (1H, ddq, J=4, 0.5, 7 Hz), 4.12 (1H, d, J=10.5 Hz), 4.57 (1H, dd, J=3.5, 4.0 Hz), 4.93—5.0 (2H, m), 5.83 (1H, d, J=3.5 Hz). MS *m/z* (relative intensity): 198 (M^+, 4.7), 183 (25), 140 (12), 128 (15), 99 (18), 95 (27), 59 (100). Exact MS *m/z* Calcd for $C_{11}H_{18}O_3$ (M^+): 198.1256. Found: 198.1251.

6-*O*-Benzyl-3-deoxy-1,2-*O*-isopropylidene-3-*C*-methyl-α-D-allofuranose (13)——A THF solution (6 ml) of **10** (5.27 g, 26.3 mmol) was added to a stirred solution of sodium benzyloxide, prepared from NaH (0.942 g, 39.25 mmol) and benzyl alcohol (4.24 g, 39.35 mmol) in DMSO (13 ml) and THF (6 ml), at room temperature. After 13 h, the reaction mixture was poured into cold saturated NH_4Cl, and extracted with CH_2Cl_2. The extract was washed with water, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (4:1) as the eluent to afford **13** as a colorless oil (6.274 g, 77%) $[\alpha]_D^{17}+19°$ (c=1.40, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.16 (3H, d, J=7 Hz), 1.33 (3H, s) 1.51 (3H, s), 2.08 (1H, ddq, J=5, 10, 7 Hz), 2.42 (1H, d, J=4 Hz), 3.54 (1H, dd, J=7, 10 Hz), 3.65 (1H, dd, J=3, 10 Hz), 3.81 (1H, dd, J=5, 10 Hz), 3.82 (1H, dddd, J=3, 4, 5, 7 Hz), 4.53 (1H, d, J=12 Hz), 4.54 (1H, dd, J=4, 5 Hz), 4,58 (1H, d, J=12 Hz), 5.75 (1H, d, J=4 Hz), 7.34 (5H, s). MS *m/z* (relative intensity): 308 (M^+, 3), 293 (6), 250 (10), 157 (30), 99 (100). Exact MS *m/z* Calcd for $C_{17}H_{24}O_5$ (M^+): 308.1625. Found: 308.1621.

6-*O*-Benzyl-3,5-dideoxy-1,2-*O*-isopropylidene-3-*C*-methyl-5-methylene-α-D-*ribo*-hexofuranose (14)——PCC (3.80 g, 17.6 mmol) and powdered molecular sieves 3 Å (12 g) were added to a stirred solution of **13** (2.371 g, 7.70 mmol) in CH_2Cl_2 (80 ml) at room temperature. After 24 h, the reaction mixture was filtered, and the filtrate was

concentrated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (3 : 2) as the eluent to afford the ketone as a colorless oil (2.09 g, 89%). IR ν_{max}^{neat} cm^{-1}: 1720. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J=7 Hz), 2.33 (3H, s), 1.50 (3H, s), 1.80—2.24 (1H, m), 4.18 (2H, d, J=11 Hz), 4.42 (2H, s), 4.54 (1H, dd, J=3.5, 4.5 Hz), 4.61 (1H, s), 5.83 (1H, d, J=3.5 Hz), 7.33 (5H, s). MS *m/z* (relative intensity): 306 (M^+, 0.25), 291 (1.6), 278 (2.0), 277 (2.6), 200 (6.1), 157 (50), 120 (15), 105 (20), 99 (75), 91 (100). Exact MS *m/z* Calcd for $C_{17}H_{22}O_5$ (M^+): 306.1467. Found: 306.1474.

A 1.85 M solution of BuLi in hexane (0.35 ml, 0.56 mmol) was added to a suspension of methyltriphenylphosphonium bromide (218 mg, 0.62 mmol) in THF (1 ml) at −10 °C, and the mixture was stirred for an additional 1 h at −10 °C and then for 2 h at room temperature. Next, a solution of the ketone (85 mg, 0.28 mmol) in THF (0.5 ml) was added dropwise at −10 °C and the reaction mixture was stirred overnight at room temperature, then poured into brine, and extracted with ether. The extract was dried ($MgSO_4$) and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (6 : 1) as the eluant to give **14** as a colorless oil (72 mg, 85%). $[\alpha]_D^{17}$ +15.0° (c=3.0, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, d, J=7 Hz), 1.33 (3H, s), 1.52 (3H, s), 2.05 (1H, ddq, J=5, 10, 7 Hz), 4.01 (1H, d, J=13.5 Hz), 4.13 (1H, d, J=13.5 Hz), 4.23 (1H, d, J=10 Hz), 4.48 (1H, d, J=12 Hz), 4.54 (1H, dd, J=3.5, 5 Hz), 4.55 (1H, d, J=12 Hz), 5.24 (1H, s), 5.34 (1H, dd, J=1.5, 3 Hz), 5.79 (1H, d, J=3.5 Hz), 7.33 (5H, s). MS *m/z* (relative intensity): 289 (M^+ − 15, 4.0), 198 (14), 183 (27), 140 (9.5), 107 (25), 91 (100). Exact MS *m/z* Calcd for $C_{17}H_{21}O_4$ (M^+ − 15): 289.1440. Found: 289.1441.

6-*O*-*tert*-Butyldimethylsilyl-3-deoxy-1,2-*O*-isopropylidene-3-*C*-methyl-α-D-allofuranose (15)——*tert*-Butyldimethylsilyl chloride (8.15 g, 54.0 mmol) in CH_2Cl_2 (10 ml) was added dropwise to a stirred solution of **9** (11.68 g, 54.0 mmol) and imidazole (9.2 g, 135 mmol) in CH_2Cl_2 (60 ml) in an ice bath. After 1 h at room temperature, the reaction mixture was poured into saturated NH_4Cl. The organic layer was washed with brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (20 : 1) to give **15** as a colorless oil (16.1 g, 90.6%). ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.90 (9H, s), 1.20 (3H, d, J=7 Hz), 1.33 (3H, s), 1.51 (3H, s), 1.90—2.08 (1H, m), 2.54 (1H, br s), 3.60—3.82 (4H, m), 4.54 (1H, dd, J=3.5, 4.5 Hz), 5.75 (1H, d, J=3.5 Hz). MS *m/z* (relative intensity): 317 (M^+ − 15, 11), 275 (7), 217 (46), 190 (32), 157 (22), 117 (86), 99 (51), 89 (33), 75 (100). Exact MS *m/z* Calcd for $C_{15}H_{29}O_5Si$ (M^+ − 15): 317.1784. Found: 317.1777.

6-*O*-*tert*-Butyldimethylsilyl-3,5-dideoxy-1,2-*O*-isopropylidene-3-*C*-methyl-5-methylene-α-D-*ribo*-hexofuranose (16)——DMSO (8 ml) was added dropwise to a stirred solution of oxalyl chloride (3.44 ml, 39.7 mmol) in CH_2Cl_2 (100 ml) at a temperature below −50 °C. After 5 min, a solution of **15** (11.0 g, 33.1 mmol) in CH_2Cl_2 (20 ml) was added. After 2 h at −50 °C, the mixture was treated with NEt_3 (24 ml), then allowed to warm to room temperature, and poured into brine. The aqueous layer was extracted with CH_2Cl_2, and the combined organic extracts were dried ($MgSO_4$). Evaporation of the solvent *in vacuo* left an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 25) to give 6-*O*-*tert*-butyldimethylsilyl-3-deoxy-3-*C*-methyl-α-D-*ribo*-hexofuranos-5-ulose (10.5 g, 96%) as a colorless oil. IR ν_{max}^{neat} cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.08 (3H, s), 0.09 (3H, s), 0.91 (9H, s), 1.20 (3H, d, J=7.0 Hz), 1.34 (3H, s), 1.51 (3H, s), 1.91—2.10 (1H, m), 4.23 (1H, d, J=10.5 Hz), 4.25—4.59 (3H, m), 5.88 (1H, d, J=3.5 Hz). MS *m/z* (relative intensity): 315 (M^+ − 15, 6), 273 (1.5), 215 (5), 157 (5), 117 (100).

A 1.6 M hexane solution of *n*-BuLi in hexane (254 ml, 0.407 mol) was added dropwise to a stirred suspension of methyltriphenylphosphonium bromide (70 g, 0.478 mol) in THF (2.5 l) at a temperature below 0 °C in an ice-salt bath. The reaction mixture was stirred for 5 h at room temperature, then cooled again to below 0 °C, and the above ulose (58.4 g, 0.177 mol) in THF was added. The mixture was stirred for 16 h, poured into saturated NH_4Cl, and extracted with CH_2Cl_2. The extract was washed with brine, and concentrated *in vacuo* to leave an oil, which was extracted with hexane. The hexane extract was evaporated *in vacuo* and the residue was chromatographed on a silica gel column. Elution with benzene–hexane (4 : 1) gave triphenylphosphine and further elution with EtOAc afforded **16** as a colorless oil (54.9 g, 95%). $[\alpha]_D^{20}$ +27.0° (c=0.98, MeOH). IR ν_{max}^{neat} cm^{-1}: 1660. ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s), 0.91 (9H, s), 1.04 (3H, d, J=7.0 Hz), 1.34 (3H, s), 1.52 (3H, s), 1.92—2.10 (1H, m), 4.00—4.32 (3H, m), 4.56 (1H, dd, J=3.5, 4.5 Hz), 5.04—5.16 (1H, m), 5.23—5.34 (1H, m), 5.80 (1H, d, J=3.5 Hz). MS *m/z* (relative intensity): 313 (M^+ − 15, 6), 271 (7), 213 (30), 143 (100). Exact MS *m/z* Calcd for $C_{16}H_{29}O_4Si$ (M^+ − 15): 313.1837. Found: 313.1835. *Anal.* Calcd for $C_{17}H_{32}O_4Si$: C, 62.15; H, 9.82. Found: C, 62.05; H, 9.88.

3,5-Dideoxy-1,2-*O*-isopropylidene-3,5-di-*C*-methyl-β-L-talofuranose (19a) and 3,5-Dideoxy-1,2-*O*-isopropylidene-3,5-di-*C*-methyl-α-D-allofuranose (19b)——a) Hydrogenation of **14** with 10% Pd–C: An EtOAc solution (8 ml) of **14** (33 mg, 0.11 mmol) was hydrogenated with 10% Pd–C (20 mg) at 0 °C for 3 h under ordinary hydrogen pressure. After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* to leave an inseparable mixture of **17a** (see below) and **17b**, which was hydrogenated again in EtOAc (5 ml) with 10% Pd–C (20 mg) for 10 h at ordinary temperature and pressure. After removal of the catalyst by filtration, evaporation of the solvent left an oil, which was subjected to silica gel thin layer chromatography (TLC) to afford two fractions. The less polar fraction gave **19a** as a colorless oil (8.7 mg, 37%). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7 Hz), 1.03 (3H, d, J=7 Hz), 1.32 (3H, s), 1.51 (3H, s), 1.60—2.40 (3H, m), 3.69 (2H, d, J=6 Hz), 3.93 (1H, dd, J=2.5, 10 Hz), 4.52 (1H, t, J=4 Hz), 5.75 (1H, d, J=4 Hz). MS *m/z* (relative intensity): 201 (M^+ − 15, 31), 157 (29), 99 (53), 59 (100). Exact MS *m/z* Calcd for $C_{10}H_{17}O_4$ (M^+ − 15): 201.1128. Found: 201.1119.

The more polar fraction gave **19b** (6.7 mg, 29%). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7 Hz), 1.05 (3H, d, J=

7 Hz), 1.35 (3H, s), 1.52 (3H, s), 1.60—2.40 (3H, m), 3.68 (2H, d, J=6 Hz), 3.68 (1H, dd, J=5, 10 Hz), 4.53 (1H, t, J=4 Hz), 5.74 (1H, d, J=4 Hz). MS m/z (relative intensity): 201 (M^+-15, 32), 157 (42), 99 (63), 71 (45), 59 (100). Exact MS m/z Calcd for $C_{10}H_{17}O_4$ (M^+-15): 201.1127. Found: 201.1125.

b) Hydrogenation of **14** with 5% Rh-Al_2O_3: An EtOH solution (80 ml) of **14** (2.478 g, 8.15 mmol) was hydrogenated with 5% Rh-Al_2O_3 (1.0 g) at 8—10 °C under ordinary pressure. After removal of the catalyst by filtration, evaporation of the solvent left an oil, which was again hydrogenated with 10% Pd-C (0.5 g) in EtOH (80 ml) at ordinary temperature and pressure for 6 h. After removal of the catalyst by filtration, the filtrate was evaporated and chromatographed on a silica gel column with hexane–EtOAc (1 : 4) to afford **19a** (1.41 g, 80%) and **19b** (0.352 g, 20%).

c) Hydroboration of **12**: A THF solution (1 ml) of **12** (0.322 g, 1.63 mmol) was added to a stirred 1 M THF solution of BH_3–THF complex (6.5 ml, 6.5 mmol) at 0 °C under argon. After 1 h at 0—5 °C, the solution was successively treated with MeOH (0.8 ml), 3 N NaOH (1 ml), and 30% H_2O_2 (1 ml) at 0 °C and then brought to 50 °C for 25 min. The mixture was extracted with ether, and the extract was washed with 2 N HCl and brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with Et_2O–hexane (4 : 1) to afford a mixture of **19a** and **19b** (0.207 g, 59%). The ratio of **19a** and **19b** was determined to be 1 : 6.8 from the C-4 proton signals (3.93 ppm for **19a** and 3.68 ppm for **19b**) in the NMR spectrum.

d) Hydroboration of **14**: A THF solution of 1 M BH_3–THF (4 ml, 4 mmol) was added to a solution of **14** (1.2 g, 3.95 mmol) in THF (20 ml) at −20 °C, and the solution was stirred for 9 h at −10 °C. The excess BH_3 was decomposed with MeOH (2 ml) at −10 °C, and 4 N NaOH (1.65 ml) and 70% *tert*-BuOOH (0.6 ml) were added to the resulting mixture at −10 °C. The mixture was stirred for 0.5 h at −10 °C, then evaporated *in vacuo*, and extracted with CH_2Cl_2. The extract was washed with brine, dried ($MgSO_4$), and evaporated to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (2 : 1) to give a mixture of **20a** and **20b** (1.03 g, 82%). The ratio of **20a** and **20b** was determined to be 24 : 1 from the anomeric proton signals (5.75 for **20a** and 5.71 for **20b**) in the NMR spectrum. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, d, J=6.5 Hz), 1.32 (3H, s), 1.49 (3H, s), 1.90—2.10 (2H, m), 2.68 (1H, dd, J=3, 8.5 Hz), 3.67 (1H, dd, J=6, 9 Hz), 3.70—3.88 (3H, m), 3.92 (1H, dd, J=3, 11 Hz), 4.54 (1H, dd, J=3.5, 4.5 Hz), 4.55 (2H, s), 3.75 (1H, d, J=3.5 Hz), 7.33 (5H, s).

The mixture (20 mg, 0.06 mmol) was treated with methanesulfonyl chloride (MsCl) (14 mg, 0.12 mmol) in CH_2Cl_2 (0.2 ml) and pyridine (0.1 ml) at room temperature. After 1.5 h, the solution was diluted with CH_2Cl_2, washed with cold 2 N HCl and brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (3 : 2) to give a mixture of mesylates (19 mg, 76.5%), which (17.6 mg, 0.044 mmol) in Et_2O (0.5 ml) was reduced with LAH (45 mg, 1.18 mmol) at 0 °C for 6.5 h to give a mixture of **17a** and **17b** (9.6 mg, 71%). The mixture (9.6 mg, 0.031 mmol) was hydrogenated with 10% Pd-C (5 mg) in EtOH (0.2 ml) at room temperature for 6 h. After removal of the catalyst by filtration, the filtrate was concentrated *in vacuo*, dissolved in Et_2O, and passed through a silica gel column to give a mixture of **19a** and **19b** (6.8 mg, 100%). The ratio of **19a** and **19b** was determined to be 24 : 1 from the NMR spectrum.

e) Reduction of **21a**: A solution of **21a** (44.7 g, 0.129 mol) and TsCl (36.9 g, 0.193 mol) in pyridine (220 ml) was allowed to stand overnight at room temperature, then poured into ice-water, and extracted with CH_2Cl_2. The extract was washed with ice-cold 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated to give tosylate (61.6 g, 95%). $[\alpha]_D^{20}$ +13.3° (c=1.26, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s), 0.81 (9H, s), 1.06 (3H, d, J=7.0 Hz), 1.30 (3H, s), 1.46 (3H, s), 1.91—2.06 (2H, m), 2.44 (3H, s), 3.57 (2H, d, J=6.5 Hz), 3.81 (1H, dd, J=3.0, 10.5 Hz), 3.99 (1H, dd, J=6.0, 10.0 Hz), 4.48 (1H, t, J=4.0 Hz), 5.65 (1H, d, J=4.0 Hz), 7.34 (2H, d, J=8.0 Hz), 7.78 (2H, d, J=8.0 Hz). MS m/z (relative intensity): 485 (M^+-15, 9.9), 443 (8), 385 (5.5), 229 (100). Exact MS m/z Calcd for $C_{23}H_{37}O_7SSi$ (M^+-15): 485.2046. Found: 485.2031.

The tosylate (51.3 g, 0.102 mol) in ether (250 ml) was added to a stirred ice-cold suspension of LAH (23.3 g, 0.616 mol) in ether (230 ml). After 5 h at room temperature, EtOAc, then water (23 ml), and 15% NaOH (23 ml) were added carefully. The resulting mixture was filtered through celite and the solid was washed with ether. The combined ether layers were dried ($MgSO_4$) and evaporated *in vacuo* to give an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 2) as the eluant to give **19a** as an oil (21.0 g, 95%).

5-*C*-(*tert*-Butyldimethylsilyloxy)methyl-1,3-dideoxy-1,2-*O*-isopropylidene-3-*C*-methyl-β-L-talofuranose (21a) and 5-*C*-(*tert*-Butyldimethylsilyloxy)methyl-3,5-dideoxy-1,2,*O*-isopropylidene-3-*C*-methyl-α-D-allofuranose (21b) ——A 1 M THF solution of BH_3–THF complex (6.55 ml, 6.6 mmol) was added to a stirred solution of **16** (2.15 g, 6.6 mmol) in THF (7 ml) at 0 °C under argon. After 1 h at 0 °C, the solution was cooled to −16 °C, and treated with 4.15 N NaOH (2.6 ml) and 70% aqueous *tert*-BuOOH solution (1.0 ml). After 3 h, the reaction mixture was diluted with ether, washed with brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 16—1 : 5) to afford **21b** as a colorless oil (0.17 g, 7.5%) from the first fraction. $[\alpha]_D^{20}$ +30° (c=1.38, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (3H, s), 0.08 (3H, s), 0.90 (9H, s), 1.10 (3H, d, J=7.0 Hz), 1.32 (3H, s), 1.50 (3H, s), 1.82—2.13 (2H, m), 2.76 (1H, t, J=6.0 Hz), 3.74—4.17 (5H, m), 4.52 (1H, dd, J=3.5, 4.5 Hz), 5.74 (1H, d, J=3.5 Hz). MS m/z (relative intensity): 331 (M^+-15, 9.9), 285 (5.5), 229 (100). Exact MS m/z Calcd for $C_{16}H_{31}O_5Si$ (M^+-15): 331.1943. Found: 331.1933.

The second fraction gave **21a** as a colorless oil (1.9 g, 84%). IR ν_{max}^{neat} cm^{-1}: 3400. $[\alpha]_D^{20}$ +41° (c=1.28, MeOH).

^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.90 (9H, s), 1.06 (3H, d, J=7.0 Hz), 1.33 (3H, s), 1.50 (3H, s), 1.79—2.17 (2H, m), 2.79 (1H, dd, J=3.0, 8.5 Hz), 3.69—3.96 (5H, m), 4.55 (1H, t, J=4.0 Hz), 5.76 (1H, d, J=4.0 Hz). MS *m/z* (relative intensity): 331 (M^+-15, 6.4), 231 (12.3), 213 (8), 201 (12), 75 (100). Exact MS *m/z* Calcd for $C_{16}H_{31}O_5Si$ (M^+-15): 331.1943. Found: 331.1962.

6-*O*-Benzyl-3,5-dideoxy-1,2-*O*-isopropylidene-3,5-di-*C*-methyl-β-L-talofuranose (17a)——A solution of **19** (1.50 g, 7.13 mmol) in THF (8 ml) was added portionwise to a stirred suspension of NaH (0.24 g, 9.99 mmol) in DMSO (6 ml) was added portionwise at room temperature. After gas evolution had ceased, benzyl chloride (0.948 g, 7.49 mmol) was added. The reaction mixture was stirred for 20 h, poured into cold aqueous NH_4Cl, and extracted with ether. The extract was washed with water and brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (4 : 1) to give **17a** as a colorless oil (2.00 g, 89%). $[\alpha]_D^{19}$ +19° (*c*=1.90, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, d, J=7.0 Hz), 1.02 (3H, d, J=7.0 Hz), 1.33 (3H, s), 1.51 (3H, s), 1.77—2.08 (2H, m), 3.41 (1H, dd, J=8.0, 14 Hz), 3.55 (1H, dd, J=8.0, 14 Hz), 3.91 (1H, dd, J=2.5, 10.5 Hz), 4.52 (1H, dd, J=3.5, 4.5 Hz), 4.53 (2H, s), 5.74 (1H, d, J=3.5 Hz), 7.32 (5H, s). MS *m/z* (relative intensity): 306 (M^+, 2.3), 291 (4.3), 248 (2.3), 231 (2.6), 157 (9.0), 91 (100). Exact MS *m/z* Calcd for $C_{18}H_{26}O_4$ (M^+): 306.1833. Found: 306.1829.

6-*O*-Benzyl-3,5-dideoxy-3,5-di-*C*-methyl-L-talofuranose (28)——A solution of **17a** (20.0 g, 65.4 mmol) in THF (750 ml) and 4 N HCl (250 ml) was allowed to stand for 10.5 h at 40 °C, then neutralized with $NaHCO_3$, and evaporated *in vacuo*. The residue was extracted with CH_2Cl_2, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 1) to afford **28** as a colorless oil (14.5 g, 83%). MS *m/z* (relative intensity): 248 (M^+-18, 5.3), 107 (28), 91 (100). Exact MS *m/z* Calcd for $C_{15}H_{20}O_3$ (M^+-18): 248.1412. Found: 248.1432.

5-*O*-Benzyl-2,4-dideoxy-3-*O*-formyl-2,4-di-*C*-methyl-L-lyxose (29)——A solution of $NaIO_4$ (8.75 g, 40.9 mmol) in water (60 ml) was added to a stirred solution of **28** (7.25 g, 27.3 mmol) in MeOH (110 ml) at 0 °C. After 1 h, the reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was extracted with CH_2Cl_2, then the extract was dried ($MgSO_4$), and evaporated *in vacuo* to give **29** as an oil (6.8 g, 94.4%). IR ν_{max}^{neat} cm^{-1}: 1720. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, d, J=7.0 Hz), 1.13 (3H, d, J=7.5 Hz), 1.98—2.30 (1H, m), 2.75 (1H, ddq, J=2.5, 7.0, 7.5 Hz), 3.20 (1H, dd, J=2, 16 Hz), 3.40 (1H, d, J=16 Hz), 4.45 (2H, s), 5.42 (1H, dd, J=7.0, 7.5 Hz), 7.32 (5H, s), 8.01 (1H, s), 9.63 (1H, d, J=2.5 Hz). MS *m/z* (relative intensity): 264 (M^+, 0.3), 188 (2.4), 160 (5.1), 145 (3.3), 112 (12), 107 (8), 91 (100). Exact MS *m/z* Calcd for $C_{15}H_{20}O_4$ (M^+): 264.1361. Found: 264.1367.

(2*Z*,4*S*,5*S*,6*S*)-7-*O*-Benzyloxy-5-hydroxy-2,4,6-trimethylhept-2-enoic Acid δ-Lactone (30)——$(MeO)_2P(O)CH(Me)CO_2Me$ (2.7 g, 13.8 mmol) was added to a stirred suspension of NaH (270 mg, 11.2 mmol) in THF (50 ml) at 0 °C. After 1 h, the solution was cooled at −80 °C and then **29** (1.21 g, 4.58 mmol) in THF (20 ml) was added dropwise at below −80 °C. The solution was allowed to warm to 8 °C overnight, treated with saturated NH_4Cl, and extracted with ether. The extract was washed with water and brine, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was dissolved in MeOH (30 ml) and treated with K_2CO_3 (0.5 g) at room temperature for 1 h. The reaction mixture was neutralized with NH_4Cl (0.5 g) ad evaporated *in vacuo*. The residue was extracted with benzene, then the extract was dried (Na_2SO_4), and evaporated to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (8 : 1) to give **30** as a colorless oil (1.15 g, 92%). ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, d, J=8 Hz), 1.08 (3H, d, J=8 Hz), 1.90 (3H, dd, J=1.5, 2 Hz), 1.95—2.36 (1H, m), 1.36—2.88 (1H, m), 3.48 (1H, dd, J=6, 10 Hz), 3.63 (1H, t, J=10 Hz), 4.27 (1H, dd, J=3, 12 Hz), 4.52 (2H, s), 6.32 (1H, dq, J=4, 2 Hz), 7.10 (5H, s). MS *m/z* (relative intensity): 274 (M^+, 6.7), 214 (7.5), 161 (37), 125 (38), 91 (100). Exact MS *m/z* Calcd for $C_{17}H_{22}O_3$ (M^+): 274.1570. Found: 274.1584.

(2*S*,5*S*,6*S*)-2*H*-5,6-Dihydro-2-isopropyloxy-3,5-dimethyl-6-[1(*S*)-methyl-2-benzyloxyethyl]pyran (31)——A 1 M toluene solution of DIBAH (49.3 ml, 49.3 mmol) was added to a stirred solution of **30** (9.0 g, 32.8 mmol) in toluene (360 ml) at −80 °C. The solution was then treated with MeOH (50 ml). After 40 min, the reaction mixture was allowed to warm to room temperature, and treated with brine (250 ml) and 1 N HCl (60 ml). The resulting mixture was extracted with ether, then the extract was washed with $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated *in vacuo* to afford the crude lactol (9.0 g, 99.4%) as an oil. The lactol (9.0 g, 32.6 mmol) was treated with camphorsulfonic acid (CSA) (0.5 g, 2.15 mmol) in Me_2CHOH (90 ml) in 1 h at room temperature. After addition of NEt_3 (10 ml), the mixture was stirred for 10 min, concentrated *in vacuo*, and extracted with ether. The extract was washed with 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 10) to afford **31** as a colorless oil (10.0 g, 97%). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, d, J=7 Hz), 0.93 (3H, d, J=7 Hz), 1.16 (3H, d, J=6 Hz), 1.17 (3H, d, J=6 Hz), 1.68 (3H, t, J=2.0 Hz), 1.90—2.50 (2H, m), 3.45 (1H, dd, J=8, 16 Hz), 3.53 (1H, dd, J=8, 16 Hz), 3.64 (1H, dd, J=2, 12 Hz), 3.96 (1H, sept, J=6 Hz), 4.45 (1H, d, J=12 Hz), 4.57 (1H, d, J=12 Hz), 4.79 (1H, br s), 5.43 (1H, br s), 7.31 (5H, s). MS *m/z* (relative intensity): 318 (M^+, 1.3), 259 (6), 167 (10), 140 (50), 98 (100). Exact MS *m/z* Calcd for $C_{20}H_{30}O_3$ (M^+): 318.2196. Found: 318.2181.

(2*S*,3*R*,5*S*,6*S*)-2-Isopropyloxy-3,5-dimethyl-6-[1(*S*)-methyl-2-hydroxyethyl]tetrahydropyran (7a) and (2*S*,3*S*,5*S*,6*S*)-2-Isopropyloxy-3,5-dimethyl-6-[1(*S*)-methyl-2-hydroxyethyl]tetrahydropyran (7b)——a) Hydrogenation of **31**: A solution of **31** (2.7 g, 8.49 mmol) in EtOAc (30 ml) was hydrogenated over 10% Pd–C (0.9 g) at ordinary

temperature and pressure for 21 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to leave **32** as an oil. MS *m/z* (relative intensity): 320 (M^+, 0.3), 278 (0.5), 260 (1.7), 91 (100). Exact MS *m/z* Calcd for $C_{17}H_{24}O_2$ (M^+-60): 260.1776. Found: 260.1767.

Compound **32** was dissolved in EtOH (15 ml) and hydrogenated again over Raney Ni W-2 (10 ml of precipitate in EtOH) for 24 h. After removal of the catalyst, the filtrate was concentrated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–benzene (1 : 20) to afford **7a** as a colorless oil (1.7 g, 87%) from the first fraction. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=7 Hz), 0.83 (3H, d, J=7 Hz), 1.00 (3H, d, J=7 Hz), 1.10 (3H, d, J=6 Hz), 1.24 (3H, d, J=6 Hz), 1.50—2.04 (3H, m), 2.76 (1H, dd, J=3.5, 8 Hz), 3.50—3.84 (3H, m), 3.77 (1H, sept. J=6 Hz), 4.65 (1H, d, J=4 Hz). MS *m/z* (relative intensity): 171 (M^+-59, 43), 143 (26), 139 (19), 100 (19), 89 (40), 81 (36), 72 (74), 71 (82), 43 (100). Exact MS *m/z* Calcd for $C_{10}H_{19}O_2$ (M^+-59): 171.1385. Found: 171.1379.

The second fraction gave **7b** (0.234 g, 12%). ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, d, J=6 Hz), 1.03 (3H, d, J=7 Hz), 1.12 (3H, d, J=6 Hz), 1.23 (3H, d, J=6 Hz), 1.40—2.10 (3H, m), 3.50—3.90 (3H, m), 3.88 (1H, sept, J=6 Hz), 4.57 (1H, s). MS *m/z* (relative intensity): 230 (M^+, 0.2), 188 (10), 171 (15), 119 (22), 100 (88), 89 (55), 82 (53), 69 (63), 58 (100). Exact MS *m/z* Calcd for $C_{13}H_{26}O_3$ (M^+): 230.1882. Found: 230.1881.

b) Hydrogenation of **38** with Pd–C: A solution of **38** (6 mg, 0.0263 mmol) in EtOAc (1 ml) at 0 °C was hydrogenated with 10% Pd–C (5 mg) for 9 h. After removal of the catalyst, evaporation of the solvent left an oil, which was chromatographed on a silica gel column with hexane–benzene (1 : 1) to give a mixture of **7a** and **7b** as a colorless oil (2.8 mg, 46%). The ratio of **7a** and **7b** was determined to be 5 : 1 from the anomeric proton signals (4.66 ppm for **7a** and 4.47 ppm for **7b**) in the NMR spectrum.

c) Hydrogenation of **38** with Rh–Al_2O_3: A solution of **38** (17 mg, 0.0745 mmol) in ether (3.5 ml) was hydrogenated with Rh–Al_2O_3 (20 mg) at room temperature overnight. After removal of the catalyst, the filtrate was evaporated to leave an oil, which was dissolved in EtOAc and passed through a silica gel column to give a 24 : 1 mixture of **7a** and **7b** (7.1 mg, 99%).

3,5-Dideoxy-1,2-*O*-isopropylidene-6-(4-methoxybenzyl)-3,5-dimethyl-β-L-talofuranose (33)——Compound **19a** (2.908 g, 13.46 mmol) was treated with NaH (0.387 g, 16.13 mmol) and 4-methoxybenzyl chloride (MPMCl) (2.53 g, 16.17 mmol) in DMSO (18 ml) as described for the preparation of **17a** to give **33** as a colorless oil (1.266 g, 99%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, d, J=7 Hz), 1.02 (3H, d, J=7 Hz), 1.33 (3H, s), 1.50 (3H, s), 1.80—2.05 (2H, m), 3.39 (1H, dd, J=7.5, 9 Hz), 3.50 (1H, dd, J=6.5, 9 Hz), 3.80 (3H, s), 3.88 (1H, dd, J=2, 10 Hz), 4.43 (1H, d, J=11.5 Hz), 4.48 (1H, d, J=11.5 Hz), 4.52 (1H, dd, J=3.5, 5 Hz), 5.74 (1H, d, J=3.5 Hz), 6.86 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 336 (M^+, 2.6), 278 (1.3), 260 (1.0), 232 (1.5), 215 (1.1), 207 (3.0), 190 (4.0), 277 (2.8), 157 (8.0), 137 (52), 121 (100). Exact MS *m/z* Calcd for $C_{19}H_{28}O_5$ (M^+): 336.1936. Found: 336.1936.

6-*O*-(4-Methoxybenzyl)-3,5-dideoxy-3,5-di-*C*-methyl-L-talofuranose (34)——Compound **33** (4.50 g, 13.45 mmol) was hydrolyzed with 4 N HCl (30 ml) in dioxane (73 ml) at 45 °C for 100 min as described for the preparation of **28** to give **34** as a colorless oil (3.10 g, 78%). ^{1}H-NMR ($CDCl_3$) δ: 0.90 (0.9H, d, J=7 Hz), 0.96 (2.1H, d, J=7 Hz), 1.04 (3H, d, J=9 Hz), 1.70—2.50 (3H, m), 2.64 (0.3H, d, J=7.5 Hz), 3.16 (0.7H, d, J=3.5 Hz), 3.20—3.70 (2H, m), 3.80 (3H, s), 3.80—4.20 (2H, m), 4.45 (2H, s), 5.21 (0.7H, d, J=3.5 Hz), 5.33 (0.3H, dd, J=4, 7.5 Hz), 6.88 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 296 (M^+, 0.2), 278 (1.5), 208 (2.0), 157 (4.1), 137 (47), 121 (100). Exact MS *m/z* Calcd for $C_{16}H_{24}O_5$ (M^+): 296.1623. Found: 296.1642.

2,4-Dideoxy-3-*O*-formyl-5-*O*-(4-methoxybenzyl)-2,4-di-*C*-methyl-L-lyxose (35)——Compound **34** (0.503 g, 1.70 mmol) was oxidized as described for the preparation of **29**, and purified by chromatography on a silica gel column with EtOAc–hexane (1 : 3) to give **35** as a colorless oil (0.465 g, 93%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1715. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, d, J=7 Hz), 1.10 (3H, d, J=7 Hz), 2.00—2.40 (1H, m), 2.74 (1H, ddq, J=2.5, 7.5, 7 Hz), 3.27 (1H, dd, J=7, 10 Hz), 3.38 (1H, dd, J=5.5, 10 Hz), 3.81 (3H, s), 4.38 (2H, s), 5.41 (1H, ddd, J=1, 5, 7.5 Hz), 6.89 (2H, d, J=9 Hz), 7.24 (2H, d, J=9 Hz), 8.09 (1H, d, J=1 Hz), 9.63 (1H, d, J=2.5 Hz). MS *m/z* (relative intensity): 294 (M^+, 1.5), 190 (1.3), 175 (1.9), 137 (13), 121 (100). Exact MS *m/z* Calcd for $C_{16}H_{22}O_5$ (M^+): 294.1467. Found: 294.1480.

(2*Z*,4*S*,5*S*,6*S*)-5-Hydroxy-7-*O*-(4-methoxybenzyl)-2,4,6-trimethylhept-2-enoic Acid δ-Lactone (36)——Compound **35** (0.465 g, 1.58 mmol) was reacted with the sodium salt of $(MeO)_2P(O)CH(Me)CO_2Me$ as described for the preparation of **30** to give **36** as a colorless oil (0.392 g, 81%). ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, d, J=7 Hz), 1.05 (3H, d, J=7 Hz), 1.90 (3H, dd, J=1.5, 2 Hz), 2.00—2.15 (1H, m), 2.55—2.70 (1H, m), 3.40 (1H, dd, J=6, 9 Hz), 3.58 (1H, dd, J=8.5, 9 Hz), 3.81 (3H, s), 4.24 (1H, dd, J=2, 11 Hz), 4.42 (1H, d, J=12 Hz), 4.48 (1H, d, J=12 Hz), 6.34 (1H, dq, J=1.5, 3.5 Hz), 6.88 (2H, d, J=9 Hz), 7.25 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 304 (M^+, 2.1), 289 (0.5), 217 (5.0), 191 (22), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{24}O_4$ (M^+): 304.1674. Found: 304.1684.

(2*S*,5*S*,6*S*)-2*H*-5,6-Dihydro-2-isopropyloxy-3,5-dimethyl-6-[1(*S*)-methyl-2-(4-methoxybenzyloxy)ethyl]pyran (37)——Compound **36** (0.314 g, 1.03 mmol) was reduced with DIBAH as described for the reduction of **30** to give the lactol as a colorless oil (0.318 g, 100%). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, d, J=7 Hz), 0.91 (3H, d, J=7 Hz), 1.73 (3H, t, J=2 Hz), 1.90—2.60 (2H, m), 3.34 (1H, dd, J=6.5, 9 Hz), 3.54 (1H, dd, J=7.5, 9 Hz), 3.70 (1H, dd, J=2, 10.5 Hz), 3.81 (3H, s), 4.46 (2H, s), 5.09 (1H, br s), 5.44 (1H, br s), 6.90 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 306 (M^+, 0.15), 288 (1.1), 167 (20), 152 (8), 137 (23), 121 (100), 99 (33). Exact MS *m/z* Calcd for $C_{18}H_{26}O_4$ (M^+): 306.1831. Found: 306.1846.

The above lactol (0.31 g, 0.89 mmol) was treated with iso-PrOH in the presence of CSA as described for the preparation of **31** to afford **37** as a colorless oil (0.341 g, 98%). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, d, J=7 Hz), 0.93 (3H, d, J=7 Hz), 1.16 (3H, d, J=6 Hz), 1.19 (3H, d, J=6 Hz), 1.67 (3H, t, J=1.5 Hz), 1.80—2.50 (2H, m), 3.39 (1H, dd, J=7.5, 9 Hz), 3.56 (1H, dd, J=6, 9 Hz), 3.64 (1H, dd, J=2, 10 Hz), 3.80 (3H, s), 3.94 (1H, sept, J=6 Hz), 4.39 (1H, d, J=12 Hz), 4.52 (1H, d, J=12 Hz), 4.78 (1H, br s), 5.42 (1H, br s), 6.86 (2H, d, J=9 Hz), 7.28 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 348 (M^+, 0.4), 305 (1.3), 288 (0.5), 227 (0.8), 167 (19), 121 (100). Exact MS *m/z* Calcd for $C_{21}H_{32}O_4$ (M^+): 348.2300. Found: 348.2283.

(1*R*,4*S*,5*S*,6*S*)-3,4,6-Trimethyl-2,9-dioxabicyclo[3.3.1]nonan-7-ene (39) and Its Dimer (40)——DDQ (0.197 g 0.868 mmol) was added to a stirred solution of **37** (0.252 g, 0.724 mmol) in CH_2Cl_2 (20 ml) and water (1 ml) at room temperature. After 1.5 h, the reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (4 : 1) to give **40** as a colorless oil (41.5 mg, 34%) from the first fraction. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (6H, d, J=7 Hz), 0.90 (6H, d, J=7 Hz), 1.68 (6H, t, J=2 Hz), 1.96—2.40 (4H, m), 3.26 (2H, dd, J=3, 4 Hz), 3.84 (2H, dd, J=2, 11 Hz), 4.07 (2H, dd, J=8, 11 Hz), 4.68 (2H, s), 5.43 (2H, s). MS *m/z* (relative intensity): 336 (M^+, 45), 254 (26), 167 (31), 123 (65), 109 (100). Exact MS *m/z* Calcd for $C_{20}H_{32}O_4$ (M^+): 336.2300. Found: 336.2283.

The second fraction gave **39** as a colorless oil (58.6 mg, 48%). ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, d, J=7 Hz), 1.09 (3H, d, J=7 Hz), 1.65 (3H, t, J=1.5 Hz), 2.00—2.30 (1H, m), 2.32—2.60 (3H, m), 3.50 (1H, d, J=11 Hz), 3.60 (1H, d, J=11 Hz), 3.63 (1H, dd, J=5, 12 Hz), 6.68—6.80 (1H, m). MS *m/z* (relative intensity): 168 (M^+, 15), 119 (25), 98 (22), 86 (65), 84 (100).

(2*S*,5*S*,6*S*)-2*H*-5,6-Dihydro-2-isopropyloxy-3,5-dimethyl-6-[1(*S*)-methyl-2-hydroxyethyl]pyran (38)——DDQ (16 mg, 0.07 mmol) was added to a CH_2Cl_2 solution (1.0 ml) of **37** (12 mg, 0.0344 mmol) containing Me_2CHOH (0.05 ml) and water (0.05 ml) at room temperature. After 2 h, the mixture was diluted with CH_2Cl_2, washed with aqueous $NaHCO_3$, dried ($MgSO_4$), and evaporated *in vacuo* to leave an oil, which was purified by TLC on silica gel with hexane–EtOAc (1 : 1) to give **38** as a colorless oil (6.1 mg, 78%). ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, d, J=7 Hz), 0.98 (3H, d, J=7 Hz), 1.18 (3H, d, J=6 Hz), 1.26 (3H, d, J=6 Hz), 1.58 (1H, br s), 1.68 (3H, t, J=2 Hz), 1.80—2.10 (1H, m), 2.10—2.44 (1H, m), 3.70 (1H, dd, J=2, 10 Hz), 3.82 (1H, d, J=10 Hz), 3.94 (1H, septet, J=6 Hz), 4.80 (1H, s), 5.44 (1H, br s). MS *m/z* (relative intensity): 228 (M^+, 0.3), 169 (20), 140 (28), 109 (40), 98 (100). Exact MS *m/z* Calcd for $C_{10}H_{17}O_2$ ($M^+ - 59$): 169.1228. Found: 169.1232.

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In contrast with the C-3-α-methyl compounds (**12**, **14**, **16**), the corresponding C-3-β-methyl compounds (**22**, **23**, **24**) showed rather opposite stereoselectivities in the formation of the hydroxy compounds (**25a, b**, **26a, b**, **27a, b**), probably because of steric repulsion between methyl groups in the transition structure (M-2) in Chart 3. The results are also shown in Table I. Details will be reported elsewhere.

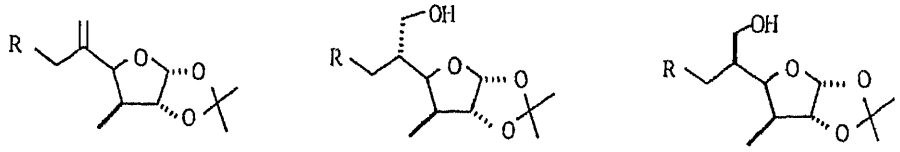

Chart 5

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20) When **37** was treated with DDQ under usual conditions for deprotection of MPM groups in dichloromethane containing a small amount of water,[10,21] a mixture of **39** and **40** instead of the desired **38** was obtained because the isopropyl protection of **38** was labile even to very weak acids. This disadvantage was overcome by the addition of isopropanol.

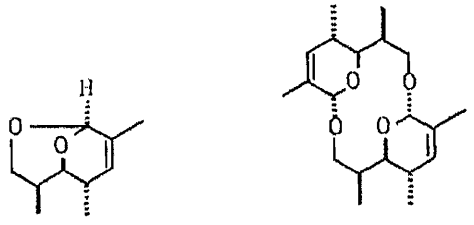

Chart 6

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Highly Stereoselective Total Synthesis of Methynolide, the Aglycon of the 12-Membered Macrolide Antibiotic Methymycin. II. Kinetic Acetalization and Synthesis of the Seco-Acid[1,2]

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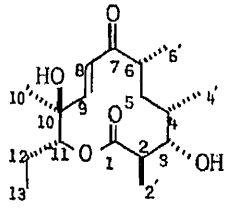
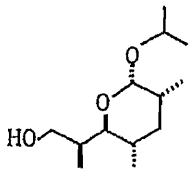
A highly stereoselective synthesis of the seco-acid (**3**) of methynolide (**1**), the aglycon of the 12-membered macrolide methymycin was carried out, starting from D-glucose *via* the Wittig–Horner coupling of the two segments i (**4**) (C-9—C-13) and ii (**5**) (C-1—C-8), which were synthesized by the use of *p*-methoxybenzyl and *p*-methoxybenzylidene acetal protecting groups for hydroxy functions.

Keywords——macrolide antibiotic; aglycon; methynolide; seco-acid; stereoselective synthesis; kinetic acetalization; protecting group; Wittig–Horner reaction

Many modern synthetic methodologies mainly consisting of means of acyclic stereocontrol, rather than classical cyclic stereocontrol, have recently been established and used to achieve marvelous total syntheses of biologically important natural products with highly complex structures such as macrolide and polyether antibiotics. Among many complex macrolides, methynolide (**1**), the aglycon of the 12-membered antibiotic methymycin, is a suitable first target in order to establish new synthetic methodologies,[3] because the structure of **1** is relatively less complex. In the preceding paper,[1] as the first step for the synthesis of **1**, we reported a highly stereoselective synthesis of a Prelog–Djerassi lactone-type versatile chiral



1



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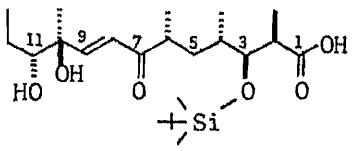

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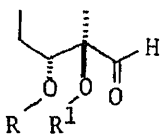
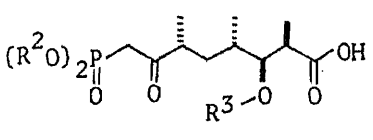


segment i



segment ii

4 : R,R^1= MP, H

5 : R^2= Et, R^3= Si

Chart 1

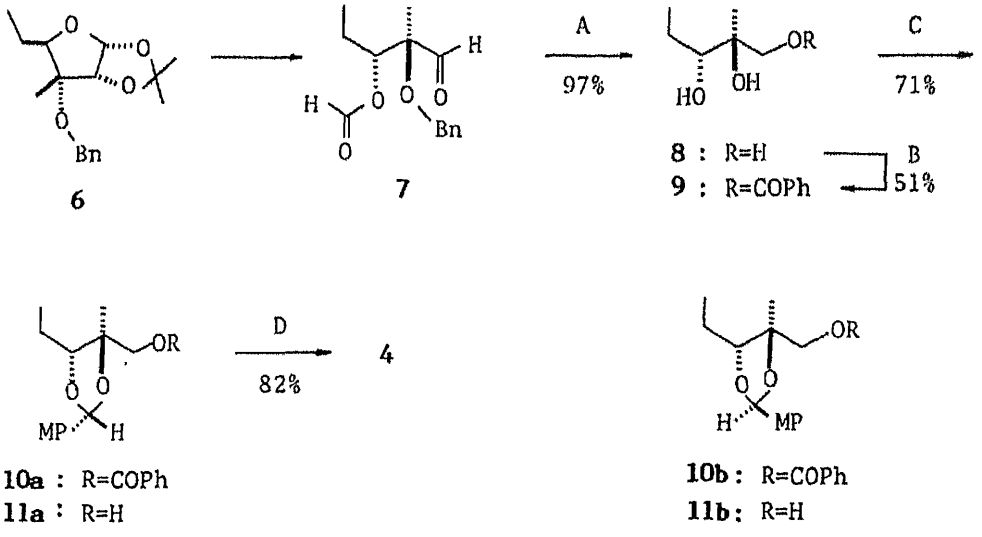
synthon (**2**), which has all the chiral centers required for segment ii and corresponds to the C-1—C-8 fragment of **1**, from D-glucose by using some stereocontrolled reactions and suitable protecting groups for hydroxy functions. In this paper we report the synthesis of both segments i (**4**) and ii (**5**) and their coupling to form the known seco-acid (**3**).

Results and Discussion

Synthesis of Segment i (4) *via* Kinetic Acetalization

The aldehyde (**7**)[4] corresponding to the segment i has already been synthesized from D-glucose *via* **6**[5] and utilized as a chiral synthon in our synthetic study of erythromycin A,[6] but **7** itself could not be used as segment i because the protecting groups of the two hydroxy functions were not suitable. It was very important to choose a protecting group for the 1,2-diol system that would be removable under conditions as mild as possible at the final synthetic step of **3**. For this purpose, *p*-methoxybenzylidene (MP acetal) protection rather than simple isopropylidene protection[7] was employed, and **7** was converted to **4** as follows.

Successive treatments of **7** with lithium aluminum hydride (LAH) and palladium on carbon (Pd–C) under a hydrogen atmosphere led to the triol (**8**), which was selectively benzoylated in pyridine to give the monobenzoate (**9**). Usual acid-catalyzed acetalization of the 1,2-diol of **9** with *p*-methoxybenzaldehyde in the presence of *p*-toluenesulfonic acid at room temperature gave a diastereoisomeric mixture of **10a** and **10b** in the ratio of 3.5 : 1.[8] Under acidic conditions two stereoisomeric acetals with respect to the benzylic carbon such as **10a** and **10b** must be in equilibrium to give a thermodynamically controlled mixture.[9,10] This disadvantage was overcome by the use of kinetically controlled acetalization with 4-methoxybenzyl methyl ether (MPMME) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ).[2a] When a mixture of **9** and MPMME in dichloromethane was reacted at room temperature for 30 min, **10a** was mainly obtained with excellent selectivity (39 : 1). Since 2,3-dichloro-5,6-dicyanohydroquinone (DDHQ) formed from DDQ is almost insoluble in the solvent,[12] the reaction mixture was kept almost neutral throughout the reaction and the acid-catalyzed equilibration would have been suppressed.[14] The benzoyl protection of **10a** was



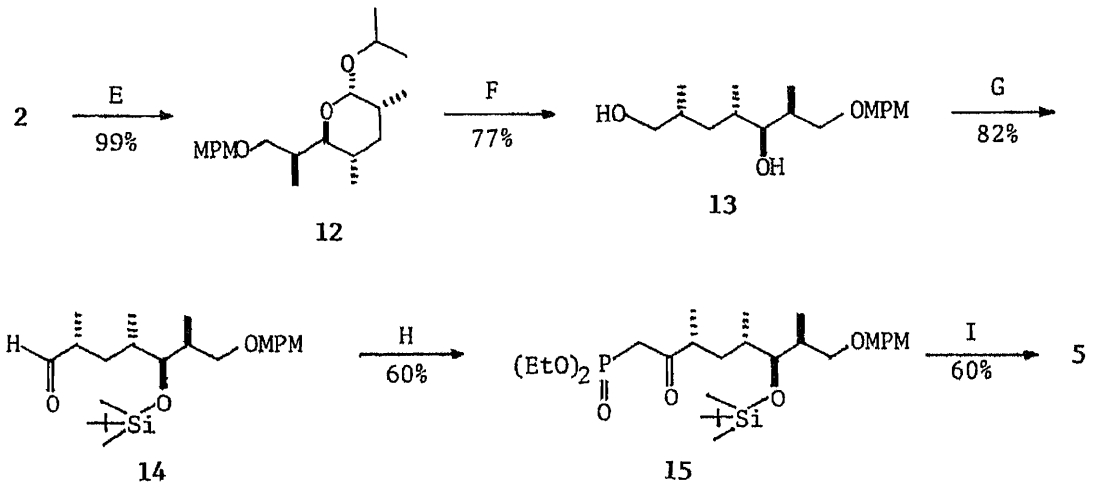
(A) 1) $LiAlH_4$, Et_2O, 0 °C; 2) Pd–C, H_2, EtOAc (B) BzCl, benzene, pyridine (C) 1) MPMME, DDQ, CH_2Cl_2; 2) KOH, MeOH (D) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −60→0 °C

Chart 2

removed with potassium hydroxide in aqueous methanol and the resulting primary alcohol (**11a**) was subjected to Swern oxidation to give the aldehyde (**4**: segment i).

Synthesis of Segment ii (5)

The hydroxy group of the Prelog–Djerassi lactone-type intermediate (**2**), synthesized highly stereoselectively from D-glucose,[1] was protected with a 4-methoxybenzyl (MPM) group[15] to give **12**, followed by acid hydrolysis of the acetal group and then calcium borohydride reduction to give the acyclic diol (**13**) in good yield. Three-step conventional conversion of **13**, *tert*-butyldimethylsilyl (TBDMS) protection of both the primary and secondary hydroxy groups, selective removal of the protection of the primary alcohol with a slight excess of a fluoride anion, and final Swern oxidation, gave the aldehyde (**14**) in high yield, and this product was treated with the lithio derivative of diethyl methylphosphonate, followed by immediate Swern oxidation of the resulting hydroxyphosphonate to give the ketophosphonate (**15**).[16] After removal of the MPM protection with DDQ in dichloromethane containing a small amount of water under usual conditions,[15] Jones oxidation of the resulting primary alcohol at 0 °C gave the carboxylic ketophosphonate (**5**: segment ii) in good yield.



12 13 14 15

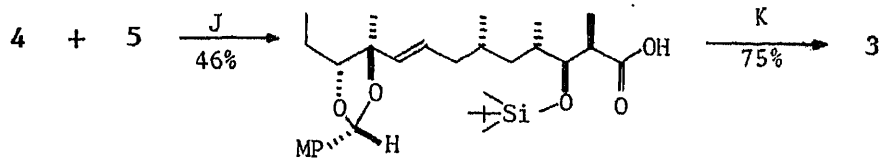
MPM = 4-MeOC$_6$H$_4$CH$_2$

(E) NaH, DMSO–THF, MPMCl (F) 1) 1 N HCl, THF, 50 °C; 2) $Ca(BH_4)_2$, EtOH (G) 1) TBDMSCl, imidazole, DMF, 90 °C; 2) Bu_4NF, THF; 3) $(COCl)_2$, DMSO–THF, Et_3N, −60→0 °C (H) 1) $(EtO)_2POMe$, *n*-BuLi, Et_2O, −78→0 °C; 2) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −60 °C (I) 1) DDQ, CH_2Cl_2–H_2O; 2) CrO_3, H_2SO_4, MeCOMe, 0 °C

Chart 3

Synthesis of Masamune's Seco-Acid (3)

The Wittig–Horner coupling[17] between the dilithio derivative of **5** and the aldehyde (**4**) proceeded smoothly in tetrahydrofuran (THF) at room temperature to afford the expected unsaturated ketone (**16**) in acceptable yield. Finally, the MP acetal protection of **16** was selectively removed without any detectable loss of the TBDMS protection by treatment with 0.4 N hydrochloric acid in dimethoxyethane at room temperature, and the expected seco-acid (**3**)[3b,c,g] was isolated in good yield.[7] Nuclear magnetic resonance (NMR) and high-resolution mass spectra of **3** were in complete agreement with those provided by Professor Ireland. Since **3** was converted to methynolide (**1**) and methymycin by Masamune *et al.*,[3b] a formal total synthesis of this macrolide antibiotic was thus completed in the present work.



16

(J) *n*-BuLi, THF (K) 0.4 N HCl, DME

Chart 4

Experimental

Physical data were measured as described in the preceding paper.[1]

(2*S*,3*R*)-2-Methylpentane-1,2,3-triol (8)——Compound 7 (0.94 g, 3.76 mmol) was reduced with $LiAlH_4$ (0.21 g, 5.53 mmol) in ether (30 ml) at 0 °C for 1 h. Work-up in the usual way and chromatography on a silica gel column with hexane–EtOAc (1 : 1) gave (2*R*,3*S*)-2-benzyloxy-2-methylpentane-1,3-diol as a colorless oil (0.813 g, 97%). $[\alpha]_D^{15}$ +19.8° (*c* = 1.0, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.06 (3H, t, *J* = 7.5 Hz), 1.07 (3H, s), 1.20—1.80 (2H, m), 2.64—2.88 (2H, m), 3.52—3.90 (3H, m), 4.55 (2H, s), 7.33 (5H, s). MS *m/z* (relative intensity): 193 (M^+ − 31, 3.0), 165 (3.5), 148 (1.8), 91 (100). Exact MS *m/z* Calcd for $C_{12}H_{17}O_2$ (M^+ − 31): 193.1228. Found: 193.1226.

An EtOAc solution (40 ml) of the diol (0.718 g, 3.21 mmol) was hydrogenated with 10% Pd–C (0.25 g) at ordinary temperature and pressure for 10 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to leave **8** as a colorless oil (0.437 g, 100%). $[\alpha]_D^{15}$ +30° (*c* = 1.16, $CHCl_3$). ^{1}H-NMR ($CDCl_3$–D_2O) δ: 1.08 (3H, t, *J* = 7 Hz), 1.20 (3H, s), 1.0—1.84 (2H, m), 3.34 (1H, dd, *J* = 2.5, 10 Hz), 3.39 (1H, d, *J* = 12 Hz), 3.52 (1H, d, *J* = 12 Hz). MS *m/z* (relative intensity): 103 (M^+ − 31, 28), 85 (13), 75 (85), 58 (80), 57 (73), 43 (100). Exact MS *m/z* Calcd for $C_5H_{11}O_2$ (M^+ − 31): 103.0759. Found: 103.0757.

(2*S*,3*R*)-1-Benzoyloxy-2-methylpentane-2,3-diol (9)——A solution of **8** (0.437 g, 3.26 mmol) and benzoyl chloride (0.55 g, 3.91 mmol) in benzene (4 ml) and pyridine (2 ml) was stirred at room temperature for 24 h. After addition of MeOH, the reaction mixture was diluted with benzene, washed with 1 N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (2 : 1) to afford **9** as a colorless oil (396 mg, 51%). ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, t, *J* = 7.5 Hz), 1.28 (3H, s), 1.20—1.92 (2H, m), 2.20—2.80 (2H, br s), 3.44 (1H, dd, *J* = 2, 10 Hz), 4.40 (1H, d, *J* = 12 Hz), 4.54 (1H, d, *J* = 12 Hz), 7.30—7.70 (3H, m), 8.40 (2H, dd, *J* = 2, 8 Hz).

(2*S*,3*R*)-2,3-[(*R*)-(4-Methoxybenzylidene)dioxy]-2-methylpentanol (11a)——A solution of **9** (0.344 g, 1.45 mmol), 4-methoxybenzyl methyl ether (0.855 g, 5.78 mmol), and DDQ (0.721 g, 3.18 mmol) in anhydrous CH_2Cl_2 (20 ml) was stirred at room temperature for 30 min. After removal of the precipitates by filtration the filtrate was washed with 5% $NaHCO_3$, dried ($MgSO_4$), and evaporated *in vacuo* to give a mixture mainly containing (2*S*,3*R*)-1-benzoyloxy-2,3-[(*R*)-(4-methoxybenzylidene)dioxy]-2-methylpentane (**10a**). ^{1}H-NMR ($CDCl_3$) δ: 1.12 (3H, t, *J* = 7 Hz), 1.47 (3H, s), 1.50—2.10 (2H, m), 3.80 (3H, s), 3.82 (1H, t, *J* = 6 Hz), 4.29 (1H, d, *J* = 12 Hz), 4.43 (1H, d, *J* = 12 Hz), 5.85 (1H, s), 6.87 (2H, d, *J* = 9 Hz), 7.44 (2H, d, *J* = 9 Hz), 7.30—7.70 (3H, m), 8.02 (2H, dd, *J* = 2.5, 8 Hz). MS *m/z* (relative intensity): 356 (M^+, 11), 355 (17), 221 (21), 137 (43), 135 (96), 105 (100). Exact MS *m/z* Calcd for $C_{21}H_{24}O_5$ (M^+): 356.1623. Found: 356.1620.

The above mixture (**10a,b**) was treated with KOH (1.0 g) in MeOH (12 ml) and water (2 ml) at room temperature for 1 h, then concentrated *in vacuo* and the residue was extracted with ether. The extract was washed with brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (3 : 1) to afford a mixture of **11a** and **11b** (0.258 g, 71%). The ratio of **11a** and **11b** was determined to be 39 : 1 from the intensity ratio of the benzylic methine protons (**11a**: 5.84 ppm and **11b**: 6.12 ppm) in the NMR spectrum. $[\alpha]_D^{18}$ +8.0° (*c* = 1.12, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, t, *J* = 7 Hz), 1.35 (3H, s), 1.50—2.00 (2H, m), 3.48 (1H, dd, *J* = 7, 12 Hz), 3.68 (1H, dd, *J* = 5, 12 Hz), 3.81 (3H, s), 3.84 (1H, t, *J* = 5 Hz), 5.84 (1H, s), 6.90 (2H, d, *J* = 8.5 Hz), 7.42 (2H, d, *J* = 8.5 Hz). MS *m/z* (relative intensity): 252 (M^+, 14), 251 (19), 221 (33), 193 (15), 137 (100), 135 (51). Exact MS *m/z* Calcd for $C_{14}H_{20}O_4$ (M^+): 252.1361. Found: 252.1344.

(2*R*,3*R*)-2,3-[(*R*)-(4-Methoxybenzylidene)dioxy]-2-methylpentanal (4)——A solution of dimethylsulfoxide (DMSO) (0.165 g, 2.11 mmol) in CH_2Cl_2 (0.35 ml) was added dropwise to a stirred solution of oxalyl chloride (0.109 g, 0.858 mmol) in CH_2Cl_2 (1.7 ml) at −60 °C, and then a solution of **11** (0.19 g, 0.753 mmol) in CH_2Cl_2 (1 ml) was similarly added. After 1 h, NEt_3 (0.254 g, 2.52 mmol) was added. The reaction mixture was allowed to warm to 0 °C during 15 min, then poured onto a silica gel column. Elution with hexane–EtOAc (3 : 1) gave **4** as a colorless oil (0.154 g, 82%). IR ν_{max}^{neat} cm^{-1}: 1740. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (3H, t, *J* = 7 Hz), 1.40 (3H, s), 1.40—1.80 (2H, m), 3.83 (3H, s), 5.99 (1H, s), 6.94 (2H, d, *J* = 9 Hz), 7.50 (2H, d, *J* = 9 Hz). MS *m/z* (relative intensity): 250 (M^+, 4), 221 (47), 152 (12), 137 (100).

(2*S*,3*R*,5*S*,6*S*)-2-Isopropyloxy-3,5-dimethyl-6-[(1*S*)-2-(4-methoxybenzyl)oxy-1-methylethyl]tetrahydropyran

(12)——A solution of **2** (0.53 g, 2.30 mmol) in THF (5 ml) was added dropwise to a stirred suspension of NaH (110 mg, 4.58 mmol) in DMSO (3 ml). After evolution of hydrogen had ceased, MPMCl (700 mg, 4.47 mmol) was added dropwise at 0 °C, and the mixture was stirred at room temperature overnight, then treated with Et_2NH (0.2 ml) at 50 °C. After 3 h, the reaction mixture was poured into cold saturated NH_4Cl and extracted with ether. The extract was washed with brine, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (5 : 1) to give **12** as a colorless oil (0.80 g, 99%). ^{1}H-NMR ($CDCl_3$) δ: 0.78 (3H, d, J=7 Hz), 0.80 (3H, d, J=7 Hz), 0.88 (3H, d, J=7 Hz), 1.07 (3H, d, J=7 Hz), 1.14 (3H, d, J=7 Hz), 1.00—2.20 (5H, m), 3.35 (1H, dd, J=7, 16 Hz), 3.50 (1H, dd, J=12, 16 Hz), 3.52 (1H, dd, J=2, 10 Hz), 3.80 (3H, s), 3.80 (1H, sept, J=7 Hz), 4.32 (1H, d, J=12 Hz), 4.48 (1H, d, J=12 Hz), 4.61 (1H, d, J=3 Hz), 6.85 (2H, d, J=9 Hz), 8.24 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 349 (M^+-1, 0.25), 290 (20), 217 (22), 169 (17), 134 (43), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{26}O_3$ (M^+-60): 290.1883. Found: 290.1882.

(2*R*,4*S*,5*S*,6*S*)-7-(4-Methoxybenzyl)oxy-2,4,6-trimethylheptane-1,5-diol (13)——A solution of **12** (800 mg, 2.28 mmol) in THF (10 ml) and 1 N HCl (3 ml) was heated at 50 °C. After 5 h, the reaction mixture was cooled, neutralized with $NaHCO_3$, and concentrated *in vacuo*. The residue was extracted with CH_2Cl_2, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (3 : 1) to give the lactol (570 mg, 81%).

A solution of $NaBH_4$ (210 mg, 5.5 mmol) in EtOH (25 ml) was added dropwise to a stirred solution of $CaCl_2$ (610 mg, 5.5 mmol) in EtOH (30 ml) at −20 °C. After 30 min, the above lactol (570 mg, 1.85 mmol) in EtOH (10 ml) was added at −20 °C, and the resulting mixture was stirred for 3 h at room temperature. Excess $Ca(BH_4)_2$ was decomposed with 1 N HCl at 0 °C and the mixture was neutralized with $NaHCO_3$. After removal of the precipitates by filtration, the filtrate was concentrated *in vacuo* and the residue was extracted with CH_2Cl_2. The extract was dried (Na_2SO_4) and evaporated *in vacuo* to leave an oil, which was dissolved in MeOH containing 1% AcOH and evaporated. This MeOH–AcOH treatment was repeated three times to decompose cyclic borates. The residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 1) to afford **13** as a colorless oil (546 mg, 95%). $[\alpha]_D^{18}$ −14.5° (c=5.68, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, d, J=7 Hz), 0.95 (6H, d, J=7 Hz), 1.40—2.10 (5H, m), 2.70 (1H, br s), 3.20 (1H, br s), 3.30—3.70 (5H, m), 3.84 (3H, s), 4.46 (2H, s), 6.88 (2H, d, J=9 Hz), 7.24 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 310 (M^+, 0.5), 292 (0.6), 171 (1.3), 150 (4.7), 137 (32), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{30}O_4$ (M^+): 310.2147. Found: 310.2140.

(2*R*,4*S*,5*S*,6*S*)-5-*tert*-Butyldimethylsilyloxy-7-(4-methoxybenzyl)oxy-2,4,6-trimethylheptanal (14)——A solution of **13** (546 mg, 1.76 mmol), TBDMS chloride (1.0 g, 6.64 mmol) and imidazole (500 mg, 7.35 mmol) in dimethylformamide (DMF) (5 ml) was stirred for 10 h at room temperature. To the resulting reaction mixture, containing the 1-monosilylate, TBDMSCl (500 mg, 3.32 mmol) and imidazole (300 mg, 4.41 mmol) were again added. The mixture was heated at 90 °C for 30 h, then cooled, poured into water (100 ml), and extracted with ether. The extract was dried (Na_2SO_4) and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (30 : 1) to give the 1,5-disilylate as a colorless oil (889 mg, 94%). ^{1}H-NMR ($CDCl_3$) δ: 0.04 (12H, s), 0.87 (3H, d, J=7 Hz), 0.89 (21H, s), 0.90 (3H, d, J=7 Hz), 1.00—2.10 (5H, m), 3.08—3.59 (4H, m), 3.62 (1H, dd, J=2, 5 Hz), 3.81 (3H, s), 4.41 (2H, s), 6.87 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 359 (M^+-151, 0.6), 323 (2.6), 227 (1.5), 187 (3.3), 121 (100). Exact MS *m/z* Calcd for $C_{19}H_{43}O_2Si_2$ (M^+-151): 359.2801. Found: 359.2790.

A solution of the disilylate (880 mg, 1.63 mmol) and a 1 M THF solution of Bu_4NF (1.8 ml, 1.8 mmol) in THF (10 ml) were mixed and allowed to stand for 10 h at room temperature, then concentrated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (5 : 1) to afford (2*R*,4*S*,5*S*,6*S*)-5-*tert*-butyldimethylsilyloxy-7-(4-methoxybenzyl)oxy-2,4,6-trimethylheptanol as a colorless oil (536 mg, 77%) from the first fraction. ^{1}H-NMR ($CDCl_3$) δ: 0.03 (3H, s), 0.07 (3H, s), 0.88 (3H, d, J=7 Hz), 0.92 (9H, s), 0.93 (3H, d, J=7 Hz), 0.95 (3H, d, J=7 Hz), 1.20—2.20 (5H, m), 3.10—3.50 (5H, m), 3.72 (1H, dd, J=2.5, 4.5 Hz), 3.84 (3H, s), 4.43 (2H, s), 6.88 (2H, d, J=9 Hz), 7.26 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 323 (M^+-101, 2.5), 187 (5.2), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{31}O_3Si$ (M^+-101): 323.2042. Found: 323.2053.

The second fraction gave the recovered starting diol (**13**: 68 mg, 13%).

A solution of DMSO (240 mg, 1.89 mmol) in CH_2Cl_2 (1.2 ml) was added dropwise to a stirred solution of $(COCl)_2$ (240 mg, 1.89 mmol) in CH_2Cl_2 (5 ml) at −60 °C, and then a solution of the above 5-monosilylate (530 mg, 1.25 mmol) in CH_2Cl_2 (1 ml) was similarly added. After 1 h, Et_3N (0.508 g, 5.03 mmol) was added and the reaction mixture was allowed to warm gradually to 0 °C, then washed with saturated NH_4Cl and water, dried ($MgSO_4$), and evaporated *in vacuo*. The resulting oil was chromatographed on a silica gel column with hexane–EtOAc (5 : 1) to give **14** as a colorless oil (513 mg, 98%). ^{1}H-NMR ($CDCl_3$) δ: 0.01 (3H, s), 0.03 (3H, s), 0.88 (9H, s), 0.90 (3H, d, J=7 Hz), 0.94 (3H, d, J=7 Hz), 1.10 (3H, d, J=7 Hz), 1.40—2.10 (5H, m), 3.20 (1H, dd, J=6, 9 Hz), 3.35 (1H, dd, J=8, 9 Hz), 3.65 (1H, dd, J=3, 5 Hz), 3.82 (3H, s), 4.41 (2H, s), 6.88 (2H, d, J=9 Hz), 7.24 (2H, d, J=9 Hz), 9.50 (1H, d, J=3 Hz). MS *m/z* (relative intensity): 323 (M^+-99, 2.1), 259 (1.1), 241 (1.0), 187 (3.4), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{31}O_3Si$ (M^+-99): 323.2042. Found: 323.2041.

(3*R*,5*S*,6*S*,7*S*)-6-*tert*-Butyldimethylsilyloxy-1-diethylphosphono-8-(4-methoxybenzyl)oxy-3,5,7-trimethyloctan-2-one (15)——A 1.6 M hexane solution of *n*-BuLi (0.85 ml, 1.36 mmol) was added to a solution of diethyl

methanephosphonate (0.320 g, 1.51 mmol) in ether (3 ml) at −78 °C under argon was added and, after 10 min, a solution of **14** (0.308 g, 0.73 mmol) in ether (3 ml) was similarly added. The reaction mixture was allowed to warm to 0 °C during 1.5 h, then treated with saturated NH_4Cl, and extracted with ether. The extract was washed with brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave the hydroxyphosphonate as a crude oil (0.403 g, 96%). MS *m/z* (relative intensity): 574 (M^+, 0.15), 517 (1.3), 379 (0.9), 363 (0.9), 321 (1.5), 295 (1.9), 181 (8), 121 (100). Exact MS *m/z* Calcd for $C_{29}H_{55}O_7PSi$ (M^+): 574.3454. Found: 574.3435.

The hydroxyphosphonate (0.403 g, 0.702 mmol) in CH_2Cl_2 (3.5 ml) was subjected to the usual Swern oxidation with $(COCl)_2$ (0.116 g, 0.916 mmol), DMSO (0.176 g, 2.26 mmol) and NEt_3 (0.218 g, 0.916 mmol) in CH_2Cl_2 (2 ml) at −60 °C. The reaction mixture was diluted with CH_2Cl_2, washed with aqueous NH_4Cl and brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 2) to give **15** as a colorless oil (0.25 g, 62%). IR ν_{max}^{neat} cm^{-1}: 1710. ^{1}H-NMR ($CDCl_3$) δ: 0.01 (3H, s), 0.04 (3H, s), 0.85 (3H, d, J=7 Hz), 0.88 (9H, s), 0.91 (3H, d, J=7 Hz), 1.15 (3H, d, J=7 Hz), 1.32 (6H, t, J=7 Hz), 1.50—2.00 (5H, m), 2.84—3.00 (1H, m), 3.01 (1H, dd, J=14, 22 Hz), 3.15 (1H, dd, J=14, 22 Hz), 3.23 (1H, dd, J=8, 9 Hz), 3.35 (1H, dd, J=6, 9 Hz), 3.64 (1H, dd, J=2.5, 5 Hz), 3.81 (3H, s), 4.13 (4H, quint, J=7 Hz), 4.41 (2H, s), 6.87 (2H, d, J=9 Hz), 7.25 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 515 (M^+ − 57, 0.3), 121 (100). Exact MS *m/z* Calcd for $C_{25}H_{44}O_7PSi$ (M^+ − 57): 515.2593. Found: 515.2606.

(2*R*,3*S*,4*S*,6*R*)-3-*tert*-Butyldimethylsilyloxy-8-diethylphosphono-7-oxo-2,4,6-trimethyloctanoic Acid (5)——A solution of **15** (0.241 g, 0.421 mmol) and DDQ (0.132 g, 0.581 mmol) in CH_2Cl_2 (10 ml) and water (0.5 ml) was stirred for 1 h at room temperature. After removal of the precipitates, the filtrate was washed with aqueous $NaHCO_3$, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 2) to afford the alcohol (0.222 g, 81%). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (3H, s), 0.08 (3H, s), 0.83 (3H, d, J=7 Hz), 0.91 (9H, s), 0.96 (3H, d, J=6.5 Hz), 1.12 (3H, d, J=7 Hz), 1.33 (6H, dt, J=1.5, 7 Hz), 1.60—1.94 (3H, m), 2.92—3.20 (1H, m), 3.04 (1H, dd, J=13, 23 Hz), 3.20 (1H, dd, J=13, 23 Hz), 3.38 (1H, dd, J=8, 11 Hz), 3.42 (1H, dd, J=5, 11 Hz), 3.89 (1H, t, J=3.5 Hz), 4.12 (4H, quint, J=7 Hz), 4.78 (1H, br s). MS *m/z* (relative intensity): 395 (M^+ − 57, 60), 365 (25), 322 (30), 303 (22), 285 (20), 261 (84), 221 (27), 208 (95), 147 (85), 99 (70), 75 (85), 73 (100). Exact MS *m/z* Calcd for $C_{17}H_{36}O_6PSi$ (M^+ − 57): 395.2018. Found: 395.2002.

A 2.67 M solution of Jones reagent (0.2 ml, 0.534 mmol) was added to a solution of the above alcohol (0.138 g, 0.305 mmol) in acetone (12 ml) at 0 °C. After 10 min, the reaction was quenched with iso-PrOH, and the solution was diluted with water (3 ml) and extracted with ether. The extract was washed with cold brine, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 4) to afford **5** as a colorless oil (0.105 g, 74%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1710. ^{1}H-NMR ($CDCl_3$) δ: 0.09 (3H, s), 0.10 (3H, s), 0.91 (9H, s), 0.99 (3H, d, J=7 Hz), 1.33 (6H, t, J=8 Hz), 1.60—2.00 (3H, m), 2.63 (1H, quint, J=7 Hz), 2.80—3.04 (1H, m), 3.17 (2H, d, J=23.5 Hz), 3.86 (1H, dd, J=2.5, 8 Hz), 4.16 (2H, quint, J=8 Hz), 4.18 (2H, quint, J=8 Hz). MS *m/z* (relative intensity): 451 (M^+ − 15, 0.8), 409 (23), 391 (8), 261 (35), 221 (13), 208 (100), 198 (90), 79 (73). Exact MS *m/z* Calcd for $C_{17}H_{34}O_7PSi$ (M^+ − 57): 409.1811. Found: 409.1794.

(2*R*,3*S*,4*S*,6*R*,8*E*,10*S*,11*R*)-3-*tert*-Butyldimethylsilyloxy-10,11-[(*R*)-(4-methoxybenzylidene)dioxy]-2,4,6,10-tetramethyl-7-oxo-8-tridecenoic Acid (16)——A 1.6 M hexane solution of *n*-BuLi (0.031 ml, 0.05 mmol) was added to a solution of **5** (9.5 mg, 0.02 mmol) in THF (0.5 ml) at 0 °C and, after 10 min, a solution of **4** (20 mg, 0.08 mmol) in THF (0.1 ml) was similarly added. The mixture was allowed to stand for 1 h at 0 °C, then for 13 h at room temperature. After addition of 5% aqueous KH_2PO_4, the mixture was extracted with CH_2Cl_2. The extract was dried ($MgSO_4$) and evaporated *in vacuo* to leave an oil, which was purified by silica gel thin layer chromatography (TLC) with CH_2Cl_2–MeOH (24 : 1) to give **16** (5.3 mg, 46%). ^{1}H-NMR ($CDCl_3$) δ: 0.03 (3H, s), 0.05 (3H, s), 0.89 (9H, s), 0.90 (3H, d, J=7 Hz), 1.06 (3H, t, J=7 Hz), 1.08 (2H, d, J=7 Hz), 1.14 (2H, d, J=7 Hz), 1.49 (3H, s), 1.57 (2H, quint, J=7 Hz), 1.70—1.92 (1H, m), 2.50—2.84 (2H, m), 3.80—3.88 (2H, m), 3.82 (3H, s), 5.89 (1H, s), 6.41 (1H, d, J=15 Hz), 6.82 (1H, d, J=15 Hz), 6.92 (2H, d, J=9 Hz), 7.45 (1H, d, J=9 Hz). MS *m/z* (relative intensity): 562 (M^+, 0.3), 561 (0.5), 369 (55), 351 (18), 137 (45), 135 (60), 75 (100).

(2*R*,3*S*,4*S*,6*R*,8*E*,10*S*,11*R*)-3-*tert*-Butyldimethylsilyloxy-10,11-dihydroxy-2,4,6,10-tetramethyl-7-oxo-8-tridecenoic Acid (3)——A solution of **16** (4.4 mg, 0.0078 mmol) in DME (0.3 ml) and 0.4 N HCl (0.15 ml) was allowed to stand for 2 h at 18 °C, and then diluted with water (0.2 ml). After addition of NaCl (for salting-out), the mixture was extracted with CH_2Cl_2, washed with brine, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was separated by TLC on silica gel to give **3** (2.6 mg, 75%). ^{1}H-NMR ($CDCl_3$) δ: 0.10 (6H, s), 0.91 (9H, s), 0.96 (3H, d, J=7 Hz), 1.02 (3H, t, J=7 Hz), 1.11 (3H, d, J=7 Hz), 1.14 (3H, d, J=7 Hz), 1.36 (3H, s), 1.45—1.90 (5H, m), 2.50—2.88 (2H, m), 3.49 (1H, dd, J=2, 10.5 Hz), 3.81 (1H, dd, J=2, 7.5 Hz), 6.48 (2H, d, J=9 Hz), 6.89 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 408 (M^+ − 36, 1.2), 384 (2), 369 (9), 311 (7), 75 (100). Exact MS *m/z* Calcd for $C_{23}H_{44}O_6Si$ (M^+ − 75): 369.2097. Found: 369.2088.

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References and Notes

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6) Y. Oikawa, T. Nishi, and O. Yonemitsu, *J. Chem. Soc., Perkin Trans. 1*, **1985**, 7.
7) Isopropylidene protection was used in Ireland's synthesis of **3**, but the yield in the deprotection with 1 N aqueous hydrochloric acid in acetonitrile was reported to be only 20% (net yield 28%).[3g]
8) The configurations of **10a** and **10b** were tentatively determined on the basis of the chemical shifts of the benzylic methine protons to be 5.85 and 6.13 ppm, respectively.[9a]
9) *a*) N. Baggett, A. B. Foster, and J. M. Webber, *Chem. Ind.* (London), **1965**, 136; *b*) N. Baggett, J. M. Duxbury, A. B. Foster, and J. M. Webber, *J. Chem. Soc.* (*C*), **1966**, 268; *c*) E. L. Eliel and W. E. Willy, *Tetrahedron Lett.*, **1975**, 1775; *d*) W. E. Willy, G. Binsch, and E. L. Eliel, *J. Am. Chem. Soc.*, **92**, 5394 (1970).
10) This presented a problem, namely when **4** (3.5:1 mixture) was coupled with segment ii (**5**), the structural determination of the crude product (**16**) was almost impossible because of its very complicated NMR spectrum.
11) Even in the acid-catalyzed reaction between benzaldehyde and 1,4-anhydroerythritol, one isomer was initially formed and then converted to an equilibrium mixture of two isomers.[9a]
12) Solubility of DDHQ in dichloromethane: 0.4 g/l at 25 °C.[13]
13) D. Walker and J. D. Hiebert, *Chem. Rev.*, **67**, 153 (1967).
14) The following mechanistic scheme may explain this kinetic acetalization, in which the acid-catalyzed back reaction from iv to iii may be suppressed under near-neutral conditions.[2a] However, prolonged treatment with DDQ caused a slow acid-catalyzed equilibration owing to the presence of a trace of DDHQ. The ratio of **10a** and **10b** fell to 15:1 when the reaction was quenched after 1.5 h.

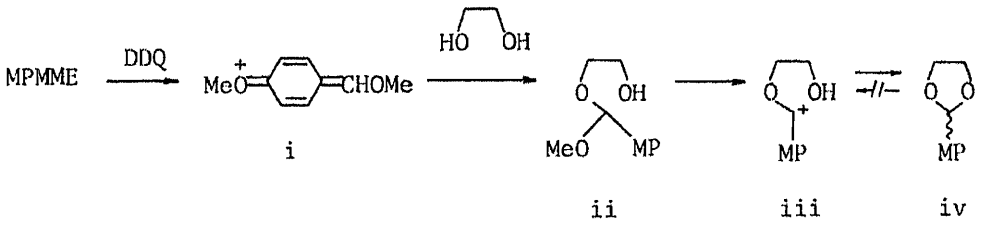

Chart 5

15) *a*) Y. Oikawa, T. Yoshioka, and O. Yonemitsu, *Tetrahedron Lett.*, **23**, 885 (1982); *b*) K. Horita, T. Yoshioka, T. Tanaka, Y. Oikawa, and O. Yonemitsu, *Tetrahedron*, **42**, 3021 (1986), and references cited therein.
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Highly Stereoselective Total Synthesis of Methynolide, the Aglycon of the 12-Membered Macrolide Antibiotic Methymycin. III. An Efficient Synthesis of Methynolide[1,2]

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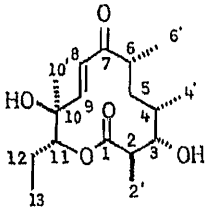
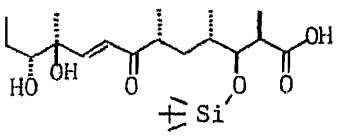
Methynolide (**1**), the aglycon of the 12-membered macrolide antibiotic methymycin, was synthesized highly stereoselectively and efficiently from D-glucose *via* two segments i (**3**: C-9—C-13) and ii (**4**: C-1—C-8). Esterification of the two segments proceeded smoothly by Yamaguchi's method. When the resulting ester (**10**) was treated with potassium carbonate in toluene in the presence of 18-crown-6 at 80 °C under Nicolaou's conditions, the intramolecular Wittig–Horner reaction occurred very smoothly, and the 12-membered cyclic enone (**11**) was isolated in excellent yield. Finally, silyl and benzyl protecting groups were removed with fluoride anion and 2,3-dichloro-5,6-dicyanobenzoquinone, respectively, to afford methynolide (**1**) in excellent yield. The overall stereoselectivity for the construction of four new chiral centers was very high (89%).

Keywords——macrolide antibiotic; methymycin; aglycon; methynolide; protecting group; esterification; macrocyclization; Wittig–Horner reaction; stereoselective synthesis

In the preceding papers,[1,3] we reported a highly stereoselective synthesis of the known seco-acid (**2**)[4] of methynolide (**1**) from D-glucose by means of some stereoselective reactions, *e.g.*, hydroboration and catalytic hydrogenation, and 4-methoxybenzyl (MPM)[5] and 4-methoxybenzylidene (MP acetal)[6] protections of hydroxy functions. The seco-acid (**2**) has already been converted to **1** by Masamune *et al. via* the macrolactonization, though the yield was unsatisfactory (20—30%).[4] Yamaguchi *et al.* also synthesized **1** using their macrolactoni-



1



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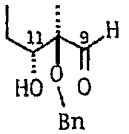
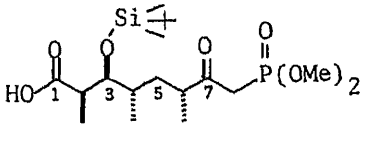


segment i

3



segment ii

4

Chart 1

zation method, which gave a better result (42% yield).[7] The seco-acid (**2**)[4] was also synthesized by Grieco *et al.*[8] and Ireland *et al.*,[9] but no conversion to **1** has been performed. Our highly stereoselective synthesis of **2**[1,3] has provided an additional example of the formal synthesis of **1**, but it was preferable to try to cyclize **2** into **1** after developing a superior macrolactonization method. Therefore, we decided to examine another synthetic route from D-glucose to **1**. In the present paper, we report an efficient synthesis of **1** *via* a key step of macrocyclization using the Wittig–Horner coupling[10] developed by Aristoff[11] and Nicolaou *et al.*[12]

Results and Discussion

We chose the aldehyde (**3**) and the carboxylic ketophosphonate (**4**) as segments i (C-9—C-13) and ii (C-1—C-8), respectively.[13]

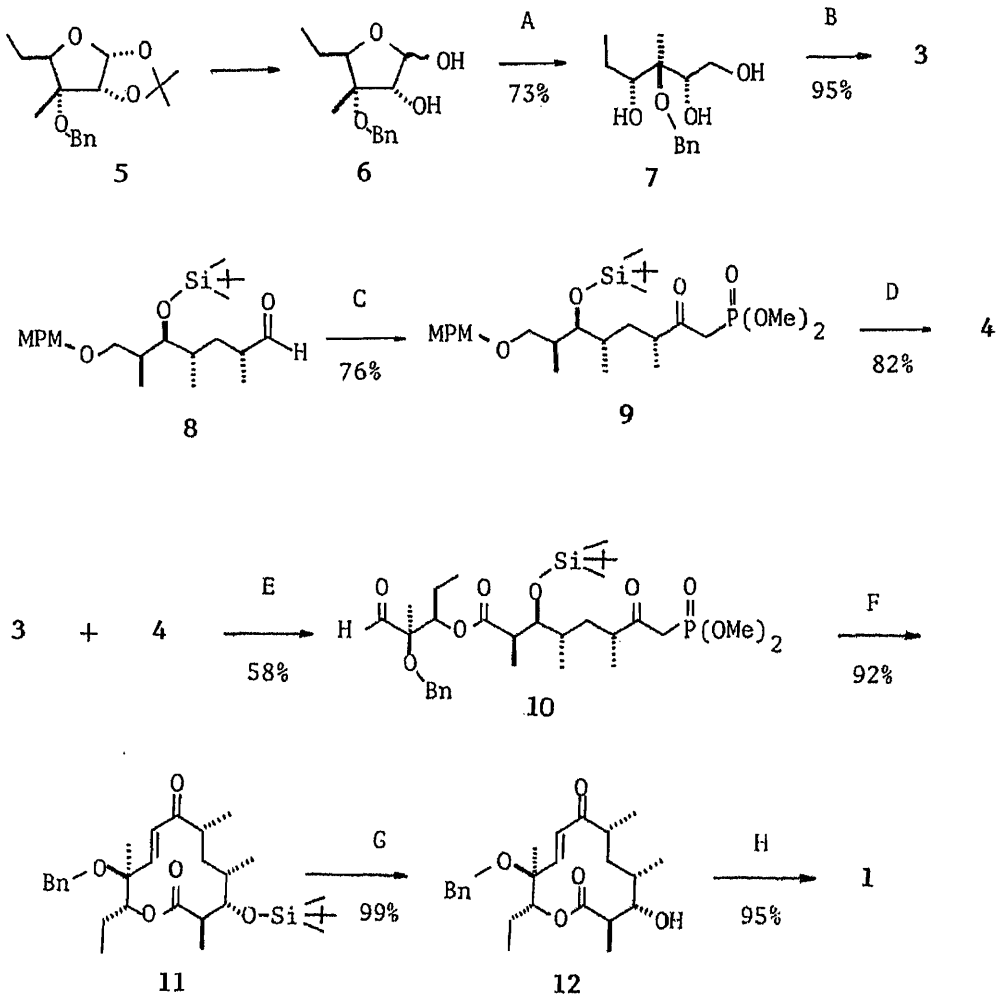
In our synthetic plan, the protecting group of the C-10 hydroxy group needed to be removed by a method other than reductive deprotection after construction of the enone structure at C-7—C-9. MPM protection, readily removable by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) oxidation,[5] was considered to be much more favorable than benzyl protection. Nevertheless, we chose the benzyl protection for the following two reasons: 1) many steps were required for the synthesis of segment i with MPM protection; 2) benzyl protection for tertiary hydroxy groups was found to be removable by prolonged treatment with DDQ.[14]

Actually, the aldehyde (**3**) bearing only two chiral centers was easily synthesized from **5**,[1,15] *via* the known hydroxy hemiacetal (**6**)[15a,c] and the acyclic triol (**7**) by only three-step conversion (acid hydrolysis, calcium borohydride reduction, and periodate oxidation) in 70% overall yield.

In the preceding paper,[1] we described the synthesis, as segment ii, of a diethyl phosphonate, whose structural confirmation was, however, somewhat troublesome because of its complex nuclear magnetic resonance (NMR) spectrum due to coupling with the phosphorus atom. Therefore, in this paper, we decided to synthesize the dimethyl analog (**4**) as segment ii from the aldehyde (**8**) by a method virtually identical with that described for the diethyl ketophosphonate in the preceding paper.[1]

Esterification of **3** and **4** with dicyclohexylcarbodiimide (DCC) in the presence of 4-dimethylaminopyridine (DMAP) proceeded rather smoothly to give the expected ester (**10**) in 70% yield, but it was very difficult to remove impurities. After examination of several esterification methods, we found that only the Yamaguchi method[16] gave an acceptable result, namely treatment of **3** and **4** with 2,4,6-trichlorobenzoyl chloride in the presence of DMAP in benzene gave pure **10**, though the reaction proceeded very slowly and over 20 h was required for disappearance of the starting materials. The ester (**10**) was then subjected to Nicolaou's macrocyclization,[12] namely when a 1 mM solution of **10** in toluene was treated with potassium carbonate (6 eq) in the presence of 18-crown-6 (12 eq) at 80 °C for 3 h,[12] a smooth cyclization proceeded to afford the expected 12-membered cyclic enone (**11**) in surprisingly high yield (92%).[17] The *tert*-butyldimethylsilyl (TBDMS) protection of **11** was first removed by treatment with fluoride anion to give **12**, and finally the benzyl group of **12** was removed; namely when **12** was treated with DDQ in dichloromethane containing a small amount of water at 40 °C, a very clean oxidative deprotection proceeded slowly, and after 9.5 h methynolide (**1**) was isolated in excellent yield. The structure of **1** was confirmed by careful examination of its 500 MHz NMR and mass spectra (MS) as well as by comparison of its physical data with those reported for an authentic sample.[4,7b]

Finally, it seems to be worth emphasizing two additional advantages of this approach, taken together with the results in the preceding two reports.[1,3] One is the very high



(A) $CaCl_2$, $NaBH_4$, EtOH (B) $NaIO_4$, MeOH–H_2O (C) 1) $MePO(OMe)_2$, *n*-BuLi, THF; 2) PDC, DMF (D) 1) DDQ, CH_2Cl_2–H_2O; 2) CrO_3, H_2SO_2, MeCOMe, 0 °C (E) 2,4,6-$Cl_3C_6H_2COCl$, Et_3N, DMAP, THF (F) K_2CO_3, 18-crown-6, toluene, 80 °C (G) *n*-Bu_4NF, THF (H) DDQ, CH_2Cl_2–H_2O, 40 °C

Chart 2

stereoselectivities throughout the synthesis of **1** from D-glucose, namely the stereoselectivities for the construction of the new chiral centers at C-2, C-4, C-6, and C-10 were 96, 97, 96, and 100%, respectively, and hence the overall stereoselectivity from D-glucose to methynolide (**1**) was 89%, which is the highest so far achieved. The other is that the methodology established here may be directly applicable to the synthesis of more complex natural products such as not only macrolides (tylonolide, pikronolide, 6-deoxyerythronolide B, erythronolide A, *etc.*) but also polyether ionophore antibiotics (salinomycin, isolasalocid A, *etc.*).

Experimental

Physical data were measured as described in the previous paper.[3]

(2*S*,3*R*,4*R*)-3-Benzyloxy-3-methyl-1,2,4-trihydroxyhexane (7)——An EtOH solution (20 ml) of sodium borohydride (300 mg, 7.8 mmol) was added dropwise to a chilled stirred EtOH solution (30 ml) of calcium chloride (840 mg, 7.6 mmol) at −20 °C. After 30 min, an EtOH solution of **6** (611 mg, 2.4 mmol) was added to the resulting calcium borohydride solution, stirring was continued for 1.5 h at room temperature, and then the excess reductant was decomposed with aqueous HCl. The mixture was neutralized with Na_2CO_3, and a white precipitate was removed by

filtration. The filtrate was concentrated *in vacuo*, and the residue was taken up in CH_2Cl_2 and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was chromatographed on a silica gel column with EtOAc to give **7** (450 mg, 73%). Leaflets, mp 69.5—70 °C (hexane–EtOAc). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 3400. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, t, J=7 Hz), 1.35 (3H, s), 1.45 (1H, ddq, J=14, 10.5, 7 Hz), 1.74 (1H, ddq, J=14, 2, 7 Hz), 2.75 (1H, d, J=4 Hz), 2.6—3.2 (1H, br s), 3.71 (1H, dd, J=10.5, 2 Hz), 3.75 (1H, dd, J=11, 4.5 Hz), 3.83 (1H, br q, J=4.5 Hz), 3.91 (1H, dd, J=11, 4.5 Hz), 4.53 (1H, d, J=12 Hz), 4.59 (1H, d, J=12 Hz), 7.25—7.4 (5H, m). MS *m/z* (relative intensity): 193 (M^+ −61, 3.7), 91 (100), 43 (11). *Anal.* Calcd for $C_{14}H_{22}O_4$: C, 66.12; H, 8.72. Found: C, 66.11; H, 8.89.

(2*R*,3*R*)-2-Benzyloxy-3-hydroxy-2-methylpentanal (3)——An aqueous solution (1 ml) of sodium metaperiodate (50 mg, 0.23 mmol) was added to a stirred MeOH solution (2 ml) of **7** (40 mg, 0.16 mmol) at room temperature. After 1 h, the reaction mixture was filtered, and the filtrate was concentrated *in vacuo* to leave an oil, which was dissolved in CH_2Cl_2. The CH_2Cl_2 solution was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was chromatographed on a silica gel column with *n*-hexane–EtOAc to give **3** as a colorless oil (33.1 mg, 95%). ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7.5 Hz), 1.21—1.67 (2H, m), 1.36 (3H, s), 2.52 (1H, d, J=3.0 Hz), 3.70 (1H, dt, J=10.0, 3.0 Hz), 4.48 (1H, d, J=11.0 Hz), 4.56 (1H, d, J=11.0 Hz), 7.2—7.5 (5H, m), 9.79 (1H, s). IR ν_{max}^{neat} cm^{-1}: 3400, 1720. $[\alpha]_D^{12}$ +82° (c=1.4, $CHCl_3$). MS *m/z* (relative intensity): 221 (M^+ −29, 3.5), 193 (1.8), 146 (1.6), 135 (2.3), 121 (3.9), 91 (100). Exact MS *m/z* Calcd for $C_{12}H_{17}O_2$ (M^+ −29): 193.1229. Found: 193.1230.

(3*R*,5*S*,6*S*,7*S*)-6-*tert*-Butyldimethylsilyloxy-1-dimethylphosphono-8-(4-methoxybenzyl)oxy-3,5,7-trimethyloctan-2-one (9)——A 1.6 M hexane solution (1.6 ml) of *n*-butyllithium was added dropwise to a stirred tetrahydrofuran (THF) solution of dimethyl methylphosphonate (387 ng, 3.1 mmol) at −78 °C. After 30 min, a THF solution (2 ml) of **8** (532 mg, 1.26 mmol) was added dropwise, and the reaction mixture was allowed to warm gradually to room temperature then poured into aqueous NH_4Cl solution, and extracted with ether. The ether extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was dissolved in dimethylformamide (DMF) (5 ml). The solution was stirred with pyridinium dichromate (PDC) (1.8 g, 4.8 mmol) for 20 h at room temperature, then poured into H_2O (100 ml), and extracted with ether. The extract was washed with brine, dried over Na_2SO_4, and evaporated to leave an oil, which was chromatographed on a silica gel column with *n*-hexane–EtOAc (1 : 1) as the eluent to give **9** as a colorless oil (523 mg, 76%). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (3H, s), 0.08 (3H, s), 0.84 (3H, d, J=7.0 Hz), 0.91 (9H, s), 0.96 (3H, d, J=7.0 Hz), 1.13 (3H, d, J=7.0 Hz), 1.13—1.29 (1H, m), 1.60—1.93 (3H, m), 2.95—3.15 (1H, m), 3.05 (1H, dd, J=23.0, 13.5 Hz), 3.21 (1H, dd, J=10, 6 Hz), 3.22 (1H, dd, J=23.0, 13.5 Hz), 3.31 (1H, dd, J=10, 8 Hz), 3.65 (1H, dd, J=5, 2.5 Hz), 3.76 (3H, d, J=11 Hz), 3.765 (3H, d, J=11 Hz), 3.80 (3H, s), 4.40 (2H, s), 6.86 (2H, d, J=9.0 Hz), 7.22 (2H, d, J=9.0 Hz). IR ν_{max}^{neat} cm^{-1}: 1710. MS *m/z* (relative intensity): 487 (M^+ −57, 0.1), 294 (1.8), 233 (4.0), 121 (100). Exact MS *m/z* Calcd for $C_{23}H_{40}O_7PSi$ (M^+ −57): 487.2280. Found: 487.2279. $[\alpha]_D^{12}$ +11° (c=1.0, $CHCl_3$).

(2*R*,3*S*,4*S*,6*R*)-3-*tert*-Butyldimethylsilyloxy-8-dimethoxyphosphono-7-oxo-2,4,6-trimethyloctanoic Acid (4)——DDQ (31 mg, 0.14 mmol) was added to a stirred CH_2Cl_2 (2 ml)–H_2O (0.1 ml) solution of **9** (50 mg, 0.092 mmol) at room temperature. After 1 h, the reaction mixture was poured into aqueous $NaHCO_3$, and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with *n*-hexane–EtOAc (1 : 2) as the eluent to give (3*R*,5*S*,6*S*,7*S*)-6-*tert*-butyldimethylsilyloxy-1-dimethylphosphono-8-hydroxy-3,5,7-trimethyloctan-2-one as a colorless oil (33 mg, 85%). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (3H, s), 0.08 (3H, s), 0.84 (3H, d, J=7.0 Hz), 0.91 (9H, s), 0.96 (3H, d, J=7.0 Hz), 1.13 (3H, d, J=7.0 Hz), 1.13—1.29 (1H, m), 1.60—1.93 (3H, m), 2.92 (1H, t, J=4.0 Hz), 2.95—3.15 (1H, m), 3.05 (1H, dd, J=23.0, 13.5 Hz), 3.22 (1H, dd, J=23.0, 13.5 Hz), 3.35—3.40 (2H, m), 3.77 (6H, d, J=12 Hz), 3.87 (1H, dd, J=3.5, 2.5 Hz). MS *m/z* (relative intensity): 425 (M^+ +1, 0.1), 409 (1.8), 367 (51), 337 (35), 233 (78), 151 (85), 147 (90), 73 (100). Exact MS *m/z* Calcd for $C_{15}H_{32}O_6PSi$ (M^+ −57): 367.1705. Found: 367.1693. IR ν_{max}^{neat} cm^{-1}: 3400, 1710. $[\alpha]_D^{12}$ +39° (c=1.1, $CHCl_3$).

A stirred ice-cold acetone solution (0.5 ml) of the above 8-hydroxy compound (28.8 mg, 0.068 mmol) was treated dropwise with 2.67 M Jones reagent (0.07 ml). After 30 min, ether (30 ml) was added, and the mixture was washed with brine (3 times), dried over anhydrous $MgSO_4$, and evaporated *in vacuo* to leave **4** as an oil (28.6 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 0.09 (3H, s), 0.10 (3H, s), 0.91 (9H, s), 0.99 (3H, d, J=6.5 Hz), 1.05—1.26 (1H, m), 1.11 (3H, d, J=7.0 Hz), 1.20 (3H, d, J=7.0 Hz), 1.64—1.84 (1H, m), 1.85 (1H, dt, J=13.5, 7.0 Hz), 2.63 (1H, dq, J=8.0, 7.0 Hz), 2.87 (1H, tq, J=7.5, 7.0 Hz), 3.19 (2H, d, J=23.0 Hz), 3.80 (3H, d, J=11.0 Hz), 3.81 (3H, d, J=11.0 Hz), 3.87 (1H, dd, J=7.5, 2.5 Hz), IR ν_{max}^{neat} cm^{-1}: 1710. MS *m/z* (relative intensity): 381 (M^+ −57, 28), 363 (23), 233 (35), 180 (100), 151 (72). Exact MS *m/z* Calcd for $C_{15}H_{30}O_7PSi$ (M^+ −57): 381.1497. Found: 381.1496.

(1*R*,2*R*)-2-Benzyloxy-1-ethyl-2-formylpropyl (2*R*,3*S*,4*S*,6*R*)-3-*tert*-Butyldimethylsilyloxy-8-dimethoxyphosphono-7-oxo-2,4,6-trimethyloctanoate (10)——2,4,6-Trichlorobenzoyl chloride (10 μl, 0.06 mmol) was added to a stirred THF solution of crude **4** (16.6 mg, 0.038 mmol) and Et_3N (10 μl, 0.07 mmol) at room temperature. After 1 h, the precipitate was filtered off, and the filtrate was evaporated *in vacuo* to leave an oil, which was taken up in benzene (2 ml). A solution of **3** (18 mg, 0.08 mmol) and DMAP (10 mg) in benzene (1 ml) was added to the above solution, and stirring was continued for 20 h at room temperature. The reaction mixture was diluted with ether, washed with brine and saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel with *n*-hexane–EtOAc (1 : 1) as the eluent to give **10** as a colorless oil (14.1 mg, 58%).

^{1}H-NMR ($CDCl_3$) δ: 0.05 (3H, s), 0.06 (3H, s), 0.89 (9H, s), 0.92 (3H, t, J=7.5 Hz), 0.96 (3H, d, J=6.0 Hz), 1.00—2.00 (5H, m), 1.11 (3H, d, J=7.0 Hz), 1.16 (3H, d, J=7.0 Hz), 1.35 (3H, s), 2.60 (1H, quint, J=7 Hz), 2.87 (1H, m), 3.03 (1H, dd, J=23, 14 Hz), 3.17 (1H, dd, J=23, 14 Hz), 3.75 (3H, d, J=11 Hz), 3.78 (3H, d, J=11 Hz), 3.86 (1H, dd, J=7.0, 3.0 Hz), 4.39 (1H, d, J=12.0 Hz), 4.60 (1H, d, J=12.0 Hz), 5.15 (1H, dd, J=9.5, 4.0 Hz), 7.33 (5H, s), 9.60 (1H, s). FI-MS m/z (relative intensity): 643 (M^++1, 41), 585 (25), 91 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720, 1710.

10-*O*-Benzyl-3-*O*-*tert*-butyldimethylsilylmethynolide (11)——A toluene solution (10 ml) of **10** (23 mg, 0.036 mmol) was added gradually to a heated suspension of anhydrous K_2CO_3 (30 mg, 0.22 mmol) in toluene (36 ml) containing 18-crown-6 (114 mg, 0.43 mmol) at 80 °C over a period of 50 min. Stirring at the same temperature was continued for an additional 2.5 h, and then, after cooling, aqueous NH_4Cl (10 ml) was added. The mixture was extracted with ether, and the ether extract was washed with saturated KCl (4 times), dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with *n*-hexane–EtOAc (5 : 1) as the eluent to give **11** as a colorless oil (17 mg, 92%). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (3H, s), 0.08 (3H, s), 0.87 (3H, t, J=7.5 Hz), 0.91 (9H, s), 0.94 (3H, d, J=7.0 Hz), 1.12—1.56 (3H, m), 1.23 (3H, d, J=7.0 Hz), 1.25 (3H, d, J=7.0 Hz), 1.44 (3H, s), 1.66 (1H, t, J=12.5 Hz), 2.00 (1H, ddq, J=14.0, 2.5, 7.5 Hz), 2.43—2.66 (1H, m), 2.63 (1H, dq, J=10.0, 7.0 Hz), 3.64 (1H, d, J=10.0 Hz), 4.43 (1H, d, J=11.5 Hz), 4.46 (1H, d, J=11.5 Hz), 4.87 (1H, dd, J=10.5, 2.5 Hz), 6.45 (1H, d, J=16.0 Hz), 6.75 (1H, d, J=16.0 Hz), 7.2—7.5 (5H, m). MS m/z (relative intensity): 459 (M^+−57, 3.4), 458 (2.0), 401 (1.9), 367 (3.7), 199 (10.5), 91 (100). FI-MS m/z (relative intensity): 517 (M^++1, 11), 461 (20), 459 (100). Exact MS m/z Calcd for $C_{26}H_{39}O_5Si$ (M^+−57): 459.2567. Found: 459.2587. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730, 1690. $[\alpha]_D^{15}$ +77° (c=0.7, $CHCl_3$).

10-*O*-Benzylmethynolide (12)——A 1 M THF solution (0.185 ml) of *n*-Bu_4NF was added to a stirred THF solution (0.5 ml) of **11** (19 mg, 0.037 mmol) at room temperature. After 24 h, the reaction mixture was evaporaed *in vacuo* to leave an oil, which was chromatographed on a silica gel column with *n*-hexane–EtOAc (3 : 1) as the eluent to give **12** as a colorless oil (14.6 mg, 99%). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7.5 Hz), 1.01 (3H, d, J=4.5 Hz), 1.23 (3H, d, J=7.0 Hz), 1.26—2.20 (6H, m), 1.33 (3H, d, J=7.0 Hz), 1.45 (3H, s), 2.43—2.82 (2H, m), 3.59 (1H, dd, J=10.5, 5.5 Hz), 4.45 (2H, s), 4.88 (1H, dd, J=10.5, 2.0 Hz), 6.40 (1H, d, J=16.0 Hz), 6.72 (1H, d, J=16.0 Hz), 7.32 (5H, s). MS m/z (relative intensity): 344 (M^+−58, 13), 253 (18), 91 (100). Exact MS m/z Calcd for $C_{21}H_{28}O_4$ (M^+−58): 344.1987. Found: 344.1981. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 1730, 1690. $[\alpha]_D^{16}$ +61° (c=0.75, $CHCl_3$).

Methynolide (1)——A CH_2Cl_2 solution (1 ml) of **12** (5.6 mg, 0.014 mmol) and DDQ (10 mg, 0.044 mmol) containing H_2O (0.1 ml) was heated under reflux for 9.5 h. After cooling, the reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, dried over anhydrous Na_2SO_4, and then evaporated to leave an oil, which was chromatographed on a silica gel column with *n*-hexane–EtOAc (2 : 1) as the eluent to give **1** as colorless crystals (4.1 mg, 95%). Recrystallization from ether–hexane gave colorless needles, mp 162.5—163.5 °C. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.90 (3H, t, J=7.5 Hz, C-13), 1.01 (3H, d, J=6.0 Hz, C-4′), 1.15—1.40 (1H, m, C-5), 1.21 (3H, d, J=7.0 Hz, C-6′), 1.33 (3H, d, J=7.0 Hz, C-2′), 1.38 (3H, s, C-10′), 1.50—1.60 (1H, m, C-4), 1.52 (1H, ddq, J=11.0, 14.0, 7.5 Hz, C-12), 1.53 (1H, d, J=6.0 Hz, C-3 OH), 1.63 (1H, t, J=12.5 Hz, C-5), 1.94 (1H, ddq, J=2.0, 14.0, 7.5 Hz, C-12), 2.04 (1H, s, C-10 OH), 2.56 (1H, dq, J=3.0, 7.0 Hz, C-6), 2.62 (1H, dq, J=10.0, 7.0 Hz, C-2), 3.57 (1H, dd, J=6.0, 10.0 Hz, C-3), 4.78 (1H, dd, J=2.0, 11.0 Hz, C-11), 6.33 (1H, d, J=15.0 Hz, C-8), 6.59 (1H, d, J=15.0 Hz, C-9). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3450, 1730, 1690. $[\alpha]_D^{16}$ +72° (c=0.45, $CHCl_3$); +56.4° (c=0.45, MeOH). MS m/z (relative intensity): 294 (M^+−18, 0.9), 254 (39), 236 (20), 198 (33), 127 (100). Exact MS m/z Calcd for $C_{17}H_{26}O_4$ (M^+−18): 294.1831. Found: 294.1844. Calcd for $C_{14}H_{22}O_4$ (M^+−58): 254.1518. Found: 254.1505.

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17) In contrast with the macrolactonization into 16-membered macrolides, 12-membered macrolactonizations have been unsatisfactory. The present cyclization by Nicolaou's method[11,12] based on the intramolecular Wittig–Horner reaction may be most promising for the construction of 12-membered macrolide rings.

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Highly Stereoselective Total Synthesis of Tylonolide, the Aglycon of the 16-Membered Macrolide Antibiotic Tylosin. I. Construction of the C-1—C-8 Chiral Centers[1,2]

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In order to synthesize tylonolide, the aglycon of the 16-membered macrolide antibiotic tylosin, a Prelog–Djerassi lactone-type compound (4) corresponding to the C-1—C-9 segment was synthesized from D-glucose. Benzyl-type protecting groups for hydroxy functions, such as benzyl, 4-methoxybenzyl, and 3,4-dimethoxybenzyl groups, as well as some cyclic and acyclic stereocontrolled reactions, such as hydroboration, catalytic hydrogenation, and Grignard reaction, were successfully employed.

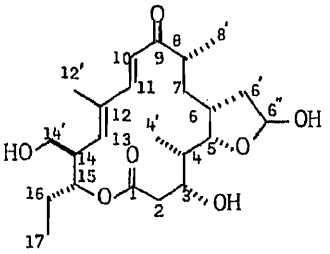
Keywords——macrolide antibiotic; tylosin; aglycon; tylonolide; chiral synthon; protecting group; acyclic stereocontrol; hydroboration; catalytic hydrogenation; Grignard reaction

In connection with our continuing interest in the area of chiral syntheses of complex polyketide-derived natural products such as macrolide and polyether ionophore antibiotics, we have extended our studies to the synthesis of tylonolide (**1**), the aglycon of a typical 16-membered ring macrolide antibiotic, tylosin, which has attracted much attention as a target molecule in current synthetic organic chemistry and is also an important therapeutic agent.[3]

In the preceding papers,[1,4] we reported a highly stereoselective synthesis of methynolide, the aglycon of the 12-membered macrolide antibiotic methymycin, from D-glucose through a synthetic methodology consisting of various means of stereochemical control in acyclic systems, selective use of suitable protecting groups, and efficient macrocyclization. This methodology was expected to be applicable for the synthesis of more complex macrolide and polyether antibiotics, and tylonolide (**1**)[5] was chosen as the next synthetic target in order to confirm its versatility. There are four precedents for the total synthesis of **1**, though the stereocontrol was quite unsatisfactory.[5] For the purpose of a highly stereoselective synthesis of **1** by our methodology, two segments i (**2**) and ii (**3**),[6] in which 4-methoxybenzyl (MPM)[7a,c] and 3,4-dimethoxybenzyl (DMPM)[7b,c] groups were used, respectively, for the protection of primary alcohols, seemed to be most suitable as intermediates.[8] In the present paper, we report the synthesis of a Prelog–Djerassi lactone-type compound (**4**) with all the chiral centers required for segment ii (**3**) from D-glucose, and in the subsequent paper, the synthesis of the two segments and tylonolide (**1**) itself will be reported.

Results and Discussion

Segment ii (**3**) has four contiguous chiral centers at C-3—C-6 and one isolated center at C-8. Since the configuration at C-4—C-8 is identical to that at C-2—C-6 of methynolide, the methodology employed in the synthesis of segment ii of methynolide[1,4] was directly applicable to the synthesis of **3**, which has only two differences, a protected hydroxyethyl group at C-6



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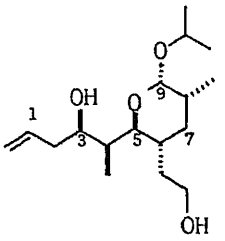

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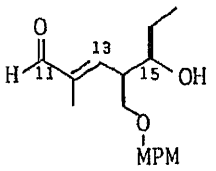
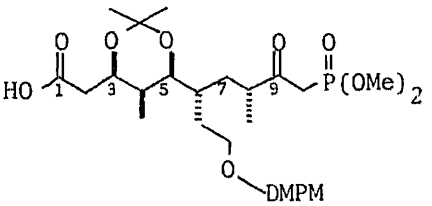


segment i

2



segment ii

3

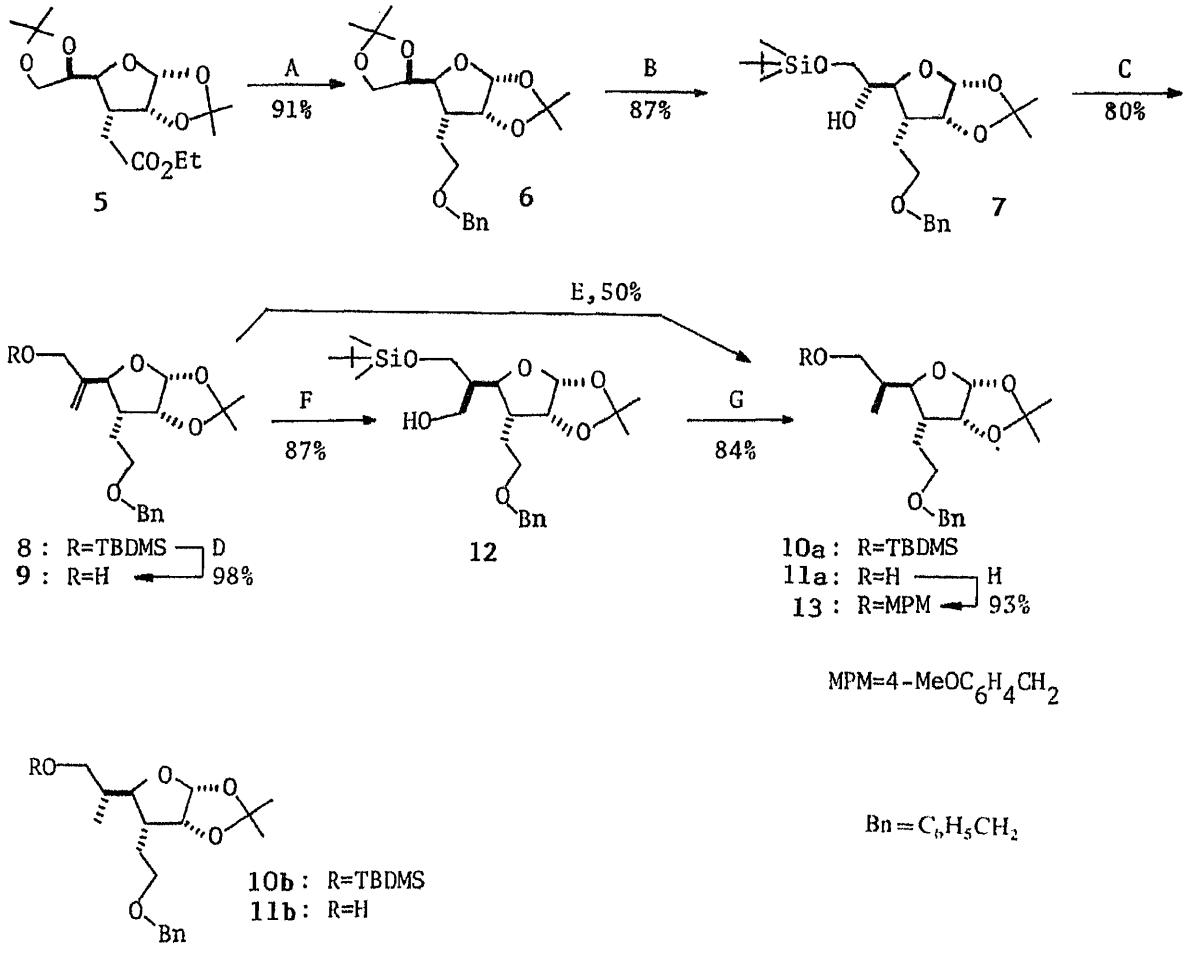
Chart 1

instead of a methyl group and an additional chiral center at C-3.

The known ester (**5**),[9] easily synthesized from D-glucose, was converted to **6** by lithium aluminum hydride (LAH) reduction followed by benzylation. The 5,6-acetonide[10] of **6** was selectively hydrolyzed with dilute sulfuric acid, and the primary alcohol of the resulting diol was selectively protected with the *tert*-butyldimethylsilyl (TBDMS) group under usual conditions to give the alcohol (**7**), which was subjected to Swern oxidation followed by Wittig reaction with a ylide prepared from methyltriphenylphosphonium bromide and *n*-butyllithium to give the olefin (**8**) in high yield.

Catalytic reduction of **8** over various catalysts, 5% platinum on carbon (Pt–C), 5% rhodium on alumina (Rh–Al_2O_3), and Wilkinson's catalyst [$(Ph_3P)_3RhCl$], gave mainly the expected product (**10a**), though the selectivity between **10a** and its isomer (**10b**) was always only about 3 : 1. The silyl protection of **8** was removed, and the resulting alcohol (**9**) was also hydrogenated, but no improvement was observed.[11] Therefore, a route *via* hydroboration was next examined. When **8** was treated with diborane in tetrahydrofuran and then with alkaline hydrogen peroxide, an acyclic stereocontrolled hydroboration[12] occurred smoothly and **12** was obtained in 92% yield[13] with 10 : 1 selectivity. The hydroboration at −30 °C gave a better result, with 87% yield and 16 : 1 selectivity. The alcohol (**12**) was tosylated, followed by LAH reduction to give **11a** in high yield. The primary alcohol of **11a** was then protected with MPM[8a,c] to give **13**. Thus the three contiguous chiral centers at C-4—C-6 of **1** were highly stereoselectively constructed.

The fourth chiral center at C-8 of **1** was introduced by the catalytic reduction of the unsaturated lactolide (**16**) as follows. The isopropylidene protection of **13** was removed with a rather strong acid, and the resulting diol was cleaved with sodium periodate to give the aldehyde (**14**), which was treated with a Wittig–Horner reagent based on trimethyl 1-phosphonopropionate[15] at −90 °C followed by treatment of the resulting (*Z*)-unsaturated ester with potassium carbonate in methanol to yield the cyclic α,β-unsaturated lactone (**15**). Although a so-called Prelog–Djerassi lactone-type compound was expected to be formed by the catalytic reduction of **15**, in order to increase the selectivity of the reduction, **15** was first

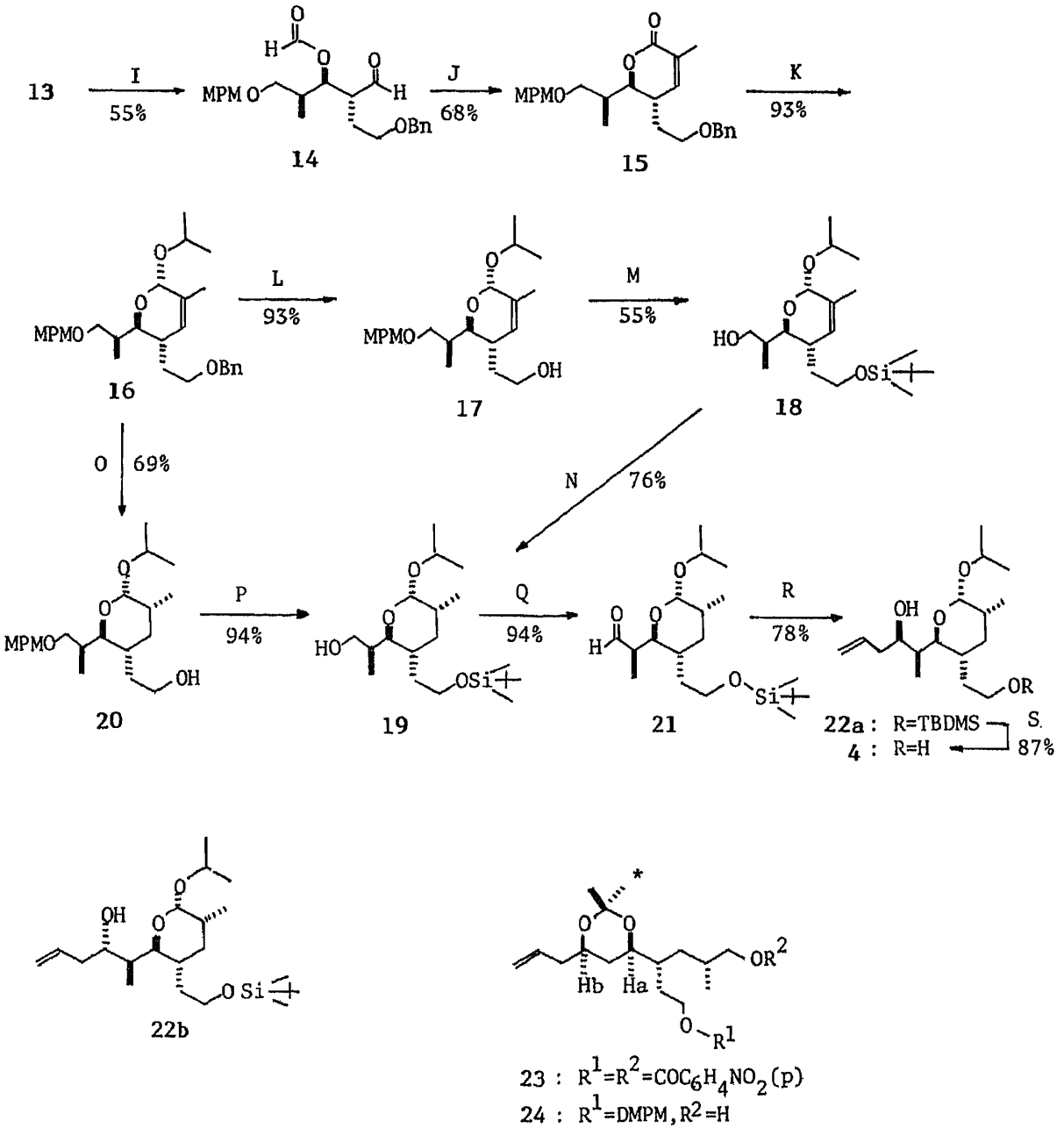


(A) 1) $LiAlH_4$, Et_2O–THF; 2) NaH, THF–DMSO, BnCl (B) 1) 2% H_2SO_4, MeOH; 2) TBDMSCl, imidazole, CH_2Cl_2, 0 °C (C) 1) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, $-70 \rightarrow 0$ °C; 2) $Ph_3P^+Me \cdot Br^-$, *n*-BuLi, THF (D) 2 N HCl, MeOH (E) Pt–C, H_2, benzene (F) B_2H_6, THF, $-70 \rightarrow -5$ °C, H_2O_2, NaOH (G) 1) TsCl, pyridine, 0 °C; 2) $LiAlH_4$, Et_2O (H) NaH, DMF–THF, MPMCl

Chart 2

converted to **16** and then subjected to the reduction. Treatment of **15** with diisobutylaluminum hydride (DIBAH) and then with camphorsulfonic acid (CSA) in isopropanol gave the anomerically pure α-lactolide (**16**) in excellent yield.[4a,16] After careful examination of various catalysts and conditions, the expected reduction product (**19**) was obtained by means of the following four-step conversion from **16**: selective removal of the benzyl protection by catalytic hydrogenation with Raney nickel (Ni)-W2 catalyst,[8c,17] TBDMS protection of the resulting alcohol (**17**), deprotection of the MPM group to give **18**,[4a] and reduction of the double bond of **18** over 5% Rh–Al_2O_3. The stereoselectivity of the reduction was 6.5 : 1.[18] However, when **16** was hydrogenated over more active Raney Ni-W4 catalyst, both the removal of the benzyl protection and the reduction of the double bond occurred simultaneously to give **20** with 6.7 : 1 selectivity. Conversion from **20** into **19** in the usual way proceeded in excellent yield.

The final chiral center at the C-3 of **1** was constructed by the Cram addition of a Grignard reagent[19] to the aldehyde (**21**), which was readily obtained from **19** by Swern oxidation. When **21** was treated with allylmagnesium bromide in ether at −90 °C, a diastereomeric mixture (6.7 : 1) of **22a** and **22b** mainly containing the Cram adduct (**22a**) was isolated in high yield.[20] After examination of several metal reagents under various conditions,



(I) 1) 4N H_2SO_4, dioxane, 65°C; 2) $NaIO_4$, MeOH–H_2O (J) 1) $MeO_2CCHMePO(OMe)_2$, NaH, THF, −90→−10°C; 2) K_2CO_3, MeOH (K) 1) DIBAH, toluene, −80°C; 2) CSA, iso-PrOH (L) Raney Ni (W-2), H_2, EtOH (M) 1) TBDMSCl, imidazole, CH_2Cl_2; 2) DDQ, CH_2Cl_2, H_2O, iso-PrOH (N) Rh–Al_2O_3, H_2, 0°C (O) Raney Ni (W-4), H_2, EtOH (P) 1) TBDMSCl, imidazole, CH_2Cl_2; 2) Pd–C, H_2, EtOAc or DDQ, CH_2Cl_2–H_2O (Q) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −80°C→room temperature (R) $CrCl_2$, $LiAlH_4$, THF, $CH_2=CHCH_2I$ (S) *n*-Bu_4NF, THF

Chart 3

the stereoselectivity was found to be slightly improved (10 : 1) by reaction with allyl iodide in the presence of chromous chloride.[21] Removal of the TBDMS protection followed by chromatographic purification gave the expected product (**4**), whose structure was confirmed after its conversion to **23** *via* **24**.[22] In the nuclear magnetic resonance (NMR) spectrum of **23**, nuclear Overhauser enhancements (NOE) were observed between the axial methyl group

TABLE I. Grignard-Type Reactions of the Aldehyde (**21**)

Reagents	Solvent	Temperature (°C)	Product	
			22a : 22b	Yield (%)
$CH_2=CH-CH_2-MgBr$	Et_2O	−90	6.7 : 1	90
$CH_2=CH-CH_2-MgBr$, CuI	Et_2O	−20	4 : 1	99
$CH_2=CH-CH_2-Li$	THF	−90	9 : 1	67
$CH_2=CH-CH_2-I$, $CrCl_2$	THF	0	10 : 1	78

(δ 1.33) (marked*) of the isopropylidene protecting group and either Ha (δ 3.61, 13%) or Hb (δ 3.81, 14%). All the chiral centers required for segment ii (**3**) were thus constructed from D-glucose. The total synthesis of tylonolide (**1**) using **4** as a chiral intermediate will be described in an accompanying paper.[23]

Experimental

Physical data were measured as described in the previous paper.[4a]

3-*C*-(2-Benzyloxyethyl)-3-deoxy-1,2 : 5,6-di-*O*-isopropylidene-α-D-allofuranose (6)——A tetrahydrofuran (THF) solution (250 ml) of **5** (40.4 g, 0.122 mol) was added dropwise to a stirred ice-cold ether solution (250 ml) of LAH (4.63 g, 0.122 mol) under an argon atmosphere. The reaction mixture was allowed to warm to room temperature, and stirring was continued overnight. The mixture was cooled again in an ice bath, and excess LAH was decomposed by the addition of MeOH (12 ml), then H_2O (5 ml), 15% NaOH (5 ml), and H_2O (5 ml). Precipitated inorganic salts were filtered off, and the filtrate was evaporated to leave a colorless oil (32.6 g), which was dissolved in THF (100 ml) and added dropwise under argon to a stirred dimethylsulfoxide (DMSO) solution (60 ml) of NaH (3.0 g, 0.125 mol). After evolution of hydrogen had ceased, $PhCH_2Cl$ (17.2 g, 0.136 mol) was added dropwise, and stirring was continued for an additional 4 h at room temperature. The reaction mixture was poured into chilled aqueous NH_4Cl, and extracted with CH_2Cl_2. The extract was washed with H_2O, dried over anhydrous $MgSO_4$, and evaporated to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (5 : 1) as the eluent to give **6** as a colorless oil (42.1 g, 91%). ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, s), 1.35 (3H, s), 1.40 (3H, s), 1.49 (3H, s), 1.70—2.25 (3H, m), 3.40—3.90 (6H, m), 4.53 (2H, s), 4.60 (1H, t, J=3.5 Hz), 5.72 (1H, d, J=3.5 Hz), 7.33 (5H, s). MS m/z (relative intensity): 378 (M^+, 0.7), 363 (3.1), 277 (2.4), 219 (3.3), 219 (3.3), 91 (100). Exact MS m/z Calcd for $C_{16}H_{21}O_4$ (M^+-101): 277.14395. Found: 271.14358. $[\alpha]_D^{24}+54.6°$ (c=1.0, $CHCl_3$).

3-*C*-(2-Benzyloxyethyl)-6-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-1,2-*O*-isopropylidene-α-D-allofuranose (7)——A stirred ice-cold MeOH solution (300 ml) of **6** (42.1 g, 0.111 mol) was treated with 2% H_2SO_4 (50 ml), and the solution was stirred for 40 h at room temperature. After neutralization with $NaHCO_3$, the reaction mixture was evaporated *in vacuo*, and the residue was extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with H_2O, dried over anhydrous $MgSO_4$, and evaporated to leave an oil (34.6 g, 0.102 mol), which was dissolved in CH_2Cl_2 (200 ml) containing imidazole (15 g, 0.224 mol). The solution was cooled in an ice-bath, and a CH_2Cl_2 solution (50 ml) of TBDMS chloride (15.7 g, 0.104 mol) was added dropwise. After 3 h, the reaction mixture was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (4 : 1) as the eluent to give **7** as a colorless oil (39.8 g, 87%). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s), 0.90 (9H, s), 1.30 (3H, s), 1.49 (3H, s), 1.70—2.25 (3H, m), 2.61 (1H, d, J=4.0 Hz), 3.40—3.90 (6H, m), 4.53 (2H, s), 4.60 (1H, t, J=3.5 Hz), 5.72 (1H, d, J=3.5 Hz), 7.33 (5H, s). MS m/z (relative intensity): 452 (M^+, 0.2), 437 (0.6), 347 (3.1), 239 (7.6), 91 (100). Exact MS m/z Calcd for $C_{24}H_{40}O_6Si$ (M^+): 452.25936. Found: 452.25996.

3-*C*-(2-Benzyloxyethyl)-6-*O*-(*tert*-butyldimethylsilyl)-3,5-dideoxy-1,2-*O*-isopropylidene-5-methylene-α-D-*ribo*-hexofuranose (8)——A CH_2CH_2 solution (50 ml) of DMSO (12.4 g, 0.159 mol) was slowly added dropwise to a stirred CH_2CH_2 solution (200 ml) of oxalyl chloride (10.6 ml, 0.102 mol) under argon at below −60 °C, and then a CH_2CH_2 solution (120 ml) of **7** (34.7 g, 76.7 mmol) was similarly added at −70 °C. After 2 h at the same temperature, Et_3N (35 ml, 0.25 mol) was added, and the reaction mixture was allowed to warm gradually to 0 °C, then washed with H_2O, cold 0.5 N HCl, cold 2% NaOCl and brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave the ketone as an oil (33.0 g, 95%). ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.92 (9H, s), 1.32 (3H, s), 1.50 (3H, s), 1.73—2.20 (3H, m), 3.57 (1H, t, J=5.5 Hz), 4.29 (1H, d, J=10.0 Hz), 4.48 (2H, s), 4.57 (2H, s), 4.63 (1H, t, J=4.0 Hz), 5.80 (1H, d, J=3.5 Hz), 7.30 (5H, s). MS m/z (relative intensity): 435 (M^+-15, 0.2), 335 (3.2), 243 (1.8), 237 (4.6), 91 (100).

A 1.6 M hexane solution of *n*-butyllithium (32.5 ml, 52 mmol) was added dropwise to a stirred ice-cold suspension of methyltriphenylphosphonium bromide (23 g, 64 mmol) in THF (200 ml), and stirring was continued for 1 h at

room temperature. The stirred solution was again cooled in an ice bath, and a THF solution (60 ml) of the above ketone (13.0 g, 29 mmol) was added dropwise. After 3 h at room temperature, the reaction mixture was poured into cold NH_4Cl solution, and extracted with ether. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (5 : 1) as the eluent to give **8** as a colorless oil (10.9 g, 84%). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s), 0.91 (9H, s), 1.32 (3H, s), 1.51 (3H, s), 1.59—2.22 (3H, m), 3.59 (2H, t, J=6.0 Hz), 4.21 (2H, s), 4.28 (1H, d, J=11 Hz), 4.51 (2H, s), 4.60 (1H, t, J=4.0 Hz), 5.12 (1H, br s), 5.30 (1H, br s), 5.78 (1H, d, J=3.5 Hz), 7.32 (5H, s), MS m/z (relative intensity): 433 (M^+-15, 0.4), 333 (3.8), 241 (2.6), 227 (3.2), 225 (4.2), 143 (34), 91 (100). Exact MS m/z Calcd for $C_{24}H_{37}O_5Si$ (M^+-15): 433.24097. Found: 433.23917. $[\alpha]_D^{20}+39°$ (c=1.1, $CHCl_3$).

3-*C*-(2-Benzyloxyethyl)-3,5-dideoxy-1,2,-*O*-isopropylidene-5-methylene-α-D-*ribo*-hexofuranose (9)——A solution of **8** (10.0 g, 22.3 mmol) in MeOH (50 ml) and 2 N HCl (10 ml) was stirred for 15 min at room temperature. After neutralization with $NaHCO_3$, the solution was evaporated *in vacuo*, and the residue was extracted with CH_2Cl_2. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (3 : 2) as the eluent to give **9** as a colorless oil (7.29 g, 98%). ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, s), 1.52 (3H, s), 1.60—2.26 (3H, m), 3.59 (2H, t, J=6.0 Hz), 4.13 (1H, d, J=10.5 Hz), 4.16 (1H, br s), 4.35 (1H, d, J=10.5 Hz), 4.50 (2H, s), 4.61 (1H, t, J=4.0 Hz), 5.12 (1H, d, J=1.0 Hz), 5.27 (1H, d, J=1.0 Hz), 5.80 (1H, d, J=3.5 Hz), 7.33 (5H, s). MS m/z (relative intensity): 334 (M^+, 0.3), 319 (1.1), 229 (1.9), 185 (2.3), 91 (100). Exact MS m/z Calcd for $C_{18}H_{23}O_5$ (M^+-15): 319.15452. Found: 319.15305.

3-*C*-(2-Benzyloxyethyl)-3,5-dideoxy-1,2-*O*-isopropylidene-5-*C*-methyl-β-L-talofuranose (11a)——(a) A benzene solution (300 ml) of **9** (4.5 g, 13.5 mmol) was hydrogenated in the presence of 5% Pt–C (400 mg) at ordinary temperature and pressure. After 3 h, the catalyst was filtered off, and the filtrate was evaporated to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–EtOAc (5 : 1) as the eluent to give two fractions. The first fraction gave 3-*C*-(2-benzyloxyethyl)-3,5-dideoxy-1,2-*O*-isopropylidene-5-*C*-methyl-α-D-allofuranose (**11b**) as a colorless oil (1.0 g, 23%). ^{1}H-NMR ($CDCl_3$) δ: 1.10 (3H, d, J=7.0 Hz), 1.30 (3H, s), 1.49 (3H, s), 1.60—2.25 (4H, m), 2.50 (1H, br s), 3.50—3.92 (5H, m), 4.52 (2H, s), 4.56 (1H, t, J=3.5 Hz), 5.74 (1H, d, J=4.0 Hz), 7.33 (5H, s). MS m/z (relative intensity): 336 (M^+, 0.35), 335 (0.85), 321 (1.9), 277 (2.2), 248 (1.4), 230 (1.2), 219 (2.3), 171 (5), 111 (9), 105 (10), 91 (100). Exact MS m/z Calcd for $C_{18}H_{25}O_5$ (M^+-15): 321.17017. Found: 321.17016.

The second fraction gave **11a** as a colorless oil (2.2 g, 50%). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7.0 Hz), 1.30 (3H, s), 1.49 (3H, s), 1.50—1.70 (2H, m), 1.71—1.90 (2H, m), 1.91—2.18 (2H, m), 3.61 (2H, dd, J=7.0, 6.0 Hz), 3.65—3.71 (2H, m), 4.01 (1H, dd, J=10.0, 2.0 Hz), 4.53 (2H, s), 4.54 (1H, t, J=4.5 Hz), 5.73 (1H, d, J=4.0 Hz). MS m/z (relative intensity): 336 (M^+, 0.1), 321 (2.2), 277 (1.8), 248 (1.8), 219 (2.2), 91 (100). Exact MS m/z Calcd for $C_{18}H_{25}O_5$ (M^+-15): 321.17017. Found: 321.16749. $[\alpha]_D^{20}+68°$ (c=1.5, $CHCl_3$).

(b) A stirred pyridine solution (40 ml) of **12** (13 g, 27.9 mmol) was treated with TsCl (12.0 g, 62.9 mmol) at room temperature. After 18 h, cold water was added. The mixture was stirred for 1 h in an ice bath, then poured into ice-water, and extracted with ether. The ether extract was washed with brine, 10% $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated *in vacuo* to leave 3-*C*-(2-benzyloxyethyl)-6-*O*-(*tert*-butyldimethylsilyl)-3,5-dideoxy-1,2-*O*-isopropylidene-5-*C*-toluenesulfonyloxymethyl-β-L-talofuranose (16.5 g, 95%). ^{1}H-NMR ($CDCl_3$) δ: 0.04 (6H, s), 0.84 (9H, s), 1.29 (3H, s), 1.46 (3H, s), 1.60—2.20 (4H, m), 2.44 (3H, s), 3.50—3.70 (4H, m), 3.78—4.35 (4H, m), 4.55 (2H, s), 5.62 (1H, d, J=3.5 Hz), 7.20—7.41 (7H, m), 7.78 (2H, d, J=8.5 Hz).

The above tosylate (16.0 g, 25.8 mmol) in ether (100 ml) was added dropwise to a stirred suspension of LAH (5.0 g, 0.132 mol) in anhydrous ether (200 ml) at room temperature. After 4 h, EtOAc was added to decompose excess LAH, then H_2O (5 ml), 15% NaOH (5 ml) and H_2O (15 ml) were added, and stirring was continued for 1 h. Precipitated inorganic salts were filtered off, and the filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 2) as eluent to give **11a** as a colorless oil (7.6 g, 88%).

3-*C*-(2-Benzyloxyethyl)-5-*C*-(*tert*-butyldimethylsilyloxy)methyl-3,5-dideoxy-1,2-*O*-isopropylidene-α-D-allofuranose (12)——A 1 M THF solution of B_2H_6 (35 ml) was added dropwise to a stirred THF solution (50 ml) of **8** (15.5 g, 34.6 mmol) at −70 °C, and the reaction mixture was allowed to gradually warm to −5 °C over a period of 6 h. After addition of MeOH to the stirred solution, 15% NaOH (7 ml) and 30% H_2O_2 (6 ml) were added at 0 °C. The reaction mixture was diluted with ether, washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 5) as the eluent to give **12** as a colorless oil (14.0 g, 87%). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (3H, s), 0.07 (3H, s), 0.89 (9H, s), 1.29 (3H, s), 1.48 (3H, s), 1.70—2.05 (3H, m), 2.06—2.20 (1H, m), 2.77 (1H, dd, J=7.0, 4.5 Hz), 3.61 (2H, t, J=6.0 Hz), 3.72—3.91 (4H, m), 3.97 (1H, dd, J=10.5, 4.0 Hz), 4.53 (2H, s), 4.55 (1H, t, J=4.0 Hz), 5.71 (1H, d, J=3.5 Hz), 7.32 (5H, s). MS m/z (relative intensity): 466 (M^+, 0.1), 451 (0.9), 351 (2,2), 243 (11), 91 (100). Exact MS m/z Calcd for $C_{25}H_{42}O_6Si$ (M^+): 466.2753. Found: 466.2726. $[\alpha]_D^{24}+45°$ (c=1.0, $CHCl_3$).

3-*C*-(2-Benzyloxyethyl)-3,5-dideoxy-1,2-*O*-isopropylidene-6-*O*-(4-methoxybenzyl)-5-*C*-methyl-β-L-talofuranose (13)——A solution of **11a** (12.5 g, 37.2 mmol) in THF (50 ml) was added dropwise to a stirred suspension of NaH (1.34 g, 55.8 mmol) in dimethylformamide (DMF) (30 ml) under argon at room temperature. After 2 h, MPM chloride (6.4 g, 40.9 mmol) was added, and stirring was continued overnight. Then Et_2NH (2 ml) was added at 50 °C. After 3 h, the reaction mixture was cooled, poured into cold aqueous NH_4Cl solution, and extracted with ether. The

extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane as the eluent to give **13** as a colorless oil (15.7 g, 93%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, d, J=7.0 Hz), 1.30 (3H, s), 1.49 (3H, s), 1.60—2.20 (4H, m), 3.39 (1H, dd, J=9.0, 4.0 Hz), 3.50 (1H, dd, J=9.0, 4.0 Hz), 3.58 (2H, t, J=7.0 Hz), 3.79 (3H, s), 3.98 (1H, dd, J=10.5, 2.5 Hz), 4.44 (2H, s), 4.52 (2H, s), 4.53 (1H, t, J=4.0 Hz), 5.71 (1H, d, J=4.0 Hz), 6.85 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz), 7.32 (5H, s). MS *m/z* (relative intensity): 398 (M^+ − 58, 6.7), 307 (1.1), 289 (1.2), 277 (2.5), 219 (2.5), 121 (100), 91 (74). Exact MS *m/z* Calcd for $C_{24}H_{30}O_5$ (M^+ − 58): 398.20929. Found: 398.20818. $[\alpha]_D^{22}$ +35° (c=1.2, $CHCl_3$).

(2*R*,3*R*,4*S*)-2-(2-Benzyloxyethyl)-3-formyloxy-5-(4-methoxybenzyloxy)-4-methylpentanal (14)——A solution of **13** (15.7 g, 34.3 mmol) in dioxane (170 ml) and 4 N H_2SO_4 (35 ml) was stirred for 4.5 h at 65 °C. After cooling, the reaction mixture was neutralized with $NaHCO_3$ and evaporated *in vacuo*. The residue was extracted with CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 1) as the eluent to give the diol as a colorless oil (9.86 g, 68%). An aqueous solution of $NaIO_4$ (7.3 g, 34 mmol) was added to a sitrred ice-cold MeOH solution (100 ml) of the above diol (6.8 g, 16.3 mmol). After 2 h, the precipitates were filtered off, then the filtrate was concentrated *in vacuo*, and extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 5) as the eluent to give **14** as a colorless oil (5.5 g, 81%). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, d, J=6.5 Hz), 1.70—2.25 (3H, m), 2.70—3.00 (1H, m), 3.20—3.35 (2H, m), 3.48 (2H, d, J=6.0 Hz), 3.79 (3H, s), 4.36 (2H, s), 4.44 (2H, s), 5.46 (1H, t, J=6.0 Hz), 6.85 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz), 7.30 (5H, s), 8.07 (1H, s), 9.65 (1H, d, J=3.0 Hz). MS *m/z* (relative intensity): 396 (M^+ − 18, 0.1), 323 (0.2), 305 (0.5), 290 (0.8), 232 (1.5), 187 (2.0), 121 (100), 91 (42). IR ν_{max}^{neat} cm^{-1}: 1725. $[\alpha]_D^{20}$ +26° (c=1.2, $CHCl_3$).

(2*Z*,4*S*,5*S*,6*S*)-4-(2-Benzyloxyethyl)-5-hydroxy-2,6-dimethyl-7-(4-methoxybenzyloxy)hept-2-enoic Acid δ-Lactone (15)——A THF solution (10 ml) of trimethyl α-phosphonopropionate (9.4 g, 48 mmol) was added dropwise to a stirred suspension of NaH (576 mg, 24 mmol) in THF (100 ml) under argon at 0 °C. After evolution of hydrogen had ceased, the solution was cooled to −90 °C, and a THF solution (20 ml) of **14** (5.5 g, 13 mmol) was added dropwise. The reaction mixture was allowed to warm to −10 °C during 5 h, and then aqueous NH_4Cl was added in order to quench the reaction. The whole mixture was extracted with ether, and the extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was dissolved in MeOH (50 ml). This solution was stirred and K_2CO_3 (1.0 g) was added at room temperature, then after 10 h NH_4Cl (1.0 g) was added. The reaction mixture was evaporated *in vacuo*, and the residue was extracted with CH_2Cl_2. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc hexane (1 : 4) as the eluent to give **15** as a colorless oil (3.82 g, 68%). ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, d, J=7.5 Hz), 1.53—1.70 (1H, m), 1.75—1.90 (1H, m), 1.88 (3H, t, J=2.0 Hz), 2.05—2.22 (1H, m), 2.63—2.83 (1H, m), 3.37 (1H, dd, J=9.5, 6.0 Hz), 3.53 (1H, t, J=9.5 Hz), 3.57 (2H, t, J=5.5 Hz), 3.80 (3H, s), 4.37 (1H, dd, J=10.0, 3.0 Hz), 4.41 (1H, s), 4.42 (1H, s), 4.48 (1H, s), 4.49 (1H, s), 6.49 (1H, q, J=1.5 Hz), 6.86 (2H, d, J=9.0 Hz), 7.18—7.39 (7H, m). MS *m/z* (relative intensity): 424 (M^+, 0.8), 333 (5.6), 191 (29), 121 (100). Exact MS *m/z* Calcd for $C_{28}H_{32}O_5$ (M^+): 424.22493. Found: 424.22590. IR ν_{max}^{neat} cm^{-1}: 1710. $[\alpha]_D^{20}$ +58° (c=1.0, $CHCl_3$).

(2*S*,5*S*,6*S*)-2-Benzyloxyethyl-5,6-dihydro-2-isopropyloxy-6-[2-(4-methoxybenzyloxy)-1(*S*)-methylethyl]-3-methyl-2*H*-pyran (16)——A 1 M hexane solution of DIBAH (12 ml) was added to a stirred toluene solution (50 ml) of **15** (4.27 g, 10.1 mmol) under argon at −80 °C. After 1 h, MeOH was added to decompose the reagent, and the reaction mixture was allowed to warm to room temperature and then extracted with ether. The ether extract was washed with 0.5 N HCl, 10% $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated to leave an oil, which was dissolved in iso-PrOH (20 ml). CSA (100 mg) was added to the above solution, and the mixture was stirred for 1 h at room temperature. After addition of Et_3N, the reaction mixture was evaporated *in vacuo*, and the residue was chromatographed on a silica gel column with EtOAc hexane (1 : 5) as the eluent to give **16** as a colorless oil (4.40 g, 93%). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, d, J=7.0 Hz), 1.15 (3H, d, J=6.0 Hz), 1.18 (3H, d, J=6.5 Hz), 1.30—1.55 (1H, m), 1.60—1.80 (1H, m), 1.67 (3H, s), 2.05—2.22 (1H, m), 2.25—2.55 (1H, m), 3.37 (1H, dd, J=9.0, 7.5 Hz), 3.52 (1H, t, J=9.0 Hz), 3.55 (2H, t, J=6.5 Hz), 3.75 (1H, dd, J=9.0, 2.0 Hz), 3.80 (3H, s), 3.94 (1H, qq, J=6.5, 6.0 Hz), 4.38 (1H, d, J=12.0 Hz), 4.46 (1H, d, J=12.0 Hz), 4.48 (2H, s), 4.77 (1H, s), 5.56 (1H, s), 6.86 (2H, d, J=9.0 Hz), 7.20—7.34 (7H, m). MS *m/z* (relative intensity): 468 (M^+, 0.2), 425 (0.3), 408 (0.8), 347 (0.8), 332 (1.2), 304 (1.3), 287 (24), 229 (10), 223 (10), 121 (100), 91 (73). Exact MS *m/z* Calcd for $C_{29}H_{40}O_5$ (M^+): 468.28753. Found: 468.28745. $[\alpha]_D^{16}$ +58° (c=1.0, $CHCl_3$).

5,6-Dihydro-5(*S*)-(2-hydroxyethyl)-2(*S*)-isopropyloxy-6(*R*)-[2-(4-methoxybenzyloxy)-1(*S*)-methyl-1(*S*)-ethyl]-3-methyl-2*H*-pyran (17)——An EtOH solution (5 ml) of **16** (135 mg, 0.29 mmol) was hydrogenated in the presence of Raney Ni W-2 (2 ml of EtOH suspension) at ordinary temperature and pressure. After 48 h, the catalyst was filtered off, and the filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 5—2 : 3) to give the recovered **16** (20.4 mg, 15%) and **17** as a colorless oil (86 mg, 79%). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7.0 Hz), 1.16 (3H, d, J=6.0 Hz), 1.19 (3H, d, J=6.5 Hz), 1.30—2.45 (5H, m), 1.69 (3H, s), 3.20—4.10 (6H, m), 4.44 (2H, ABq, J=11 Hz), 4.78 (1H, s), 5.57 (1H, s), 6.86 (2H, d, J=9.0 Hz), 7.26 (2H, d, J=9.0 Hz). MS *m/z* (relative intensity): 335 (M^+ − 43, 0.7), 320 (1.8), 259 (6), 197 (15), 147 (13), 121 (100). Exact MS *m/z*

Calcd for $C_{19}H_{27}O_5$ (M^+-43): 335.18581, Found: 335.18774.

5(*S*)-[2-(*tert*-Butyldimethylsilyloxy)ethyl]-5,6-dihydro-6(*R*)-(2-hydroxy-1(*S*)-methyl-1(*S*)-ethyl)-2(*S*)-isopropyloxy-3-methyl-2*H*-pyran (18)——TBDMS chloride (50 mg, 0.32 mmol) was added to a stirred CH_2Cl_2 solution (2 ml) of **17** (86 mg, 0.23 mmol) and imidazole (50 mg, 0.75 mmol) at room temperature. After 15 min, the reaction mixture was evaporated, and the residue was chromatographed on a silica gel column with EtOAc–hexane (1 : 15) as the eluent to give 5(*S*)-[2-(*tert*-butyldimethylsilyloxy)ethyl]-5,6-dihydro-2(*S*)-isopropyloxy-6(*R*)-[2-(4-methoxybenzyloxy)-1(*S*)-methyl-1(*S*)-ethyl]-3-methyl-2*H*-pyran as a colorless oil (108 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 0.03 (6H, s), 0.88 (9H, s), 1.19 (6H, t, J=7.0 Hz), 1.30—2.45 (4H, m), 1.70 (3H, s), 3.20—3.90 (6H, m), 3.79 (3H, s), 4.43 (2H, s), 4.69 (1H, s), 5.60 (1H, s), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0). MS *m/z* (relative intensity): 449 (M^+-43, 0.1), 338 (0.9), 311 (6.0), 253 (6.0), 121 (100). Exact MS *m/z* Calcd for $C_{25}H_{41}O_5Si$ (M^+-43): 449.27228. Found: 449.27157.

DDQ (100 mg, 0.44 mmol) was added to a stirred cold solution of the above TBDMS compound (108 mg, 0.22 mmol) in CH_2Cl_2 (4 ml) containing iso-PrOH (0.2 ml) and H_2O (0.2 ml) in an ice bath. After 25 min, the reaction mixture was poured into 10% $NaHCO_3$ solution, and extracted with CH_2Cl_2. The extract was washed with 10% $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 10) as the eluent to give **18** as a colorless oil (46 mg, 57%). ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.89 (9H, s), 0.99 (3H, d, J=7.0 Hz), 1.25 (6H, t, J=7.0 Hz), 1.30—2.45 (5H, m), 1.71 (3H, s), 3.30—4.00 (6H, m), 4.72 (1H, s), 5.30 (1H, s). MS *m/z* (relative intensity): 313 (M^+-59, 3.2), 297 (1.4), 284 (1.2), 255 (52), 185 (16), 163 (27), 75 (100). Exact MS *m/z* Calcd for $C_{17}H_{33}O_3Si$ (M^+-59): 313.21985. Found: 313.22087.

5(*R*)-(2-Hydroxyethyl)-2(*S*)-isopropyloxy-6(*S*)-[2-(4-methoxybenzyloxy)-1(*S*)-methyl-1(*S*)-ethyl]-3(*R*)-methyltetrahydropyran (20)——An EtOH solution (10 ml) of **16** (655 mg, 1.40 mmol) was hydrogenated in the presence of Raney Ni W-4 (10 ml of EtOH suspension) at ordinary temperature and pressure for 4 h. After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 3) as the eluent to give **20** as a colorless oil (366 mg, 69%). ^{1}H-NMR ($CDCl_3$) δ: 0.82 (3H, d, J=7.0 Hz), 0.88 (3H, d, J=7.0 Hz), 1.07 (3H, d, J=6.0 Hz), 1.14 (3H, d, J=6.0 Hz), 1.20—1.82 (7H, m), 2.97 (1H, m), 3.31 (1H, dd, J=9.0, 7.0 Hz), 3.47 (1H, dd, J=9.0, 7.0 Hz), 3.60—3.95 (4H, m), 3.80 (3H, s), 4.37 (1H, d, J=11.5 Hz), 4.41 (1H, d, J=11.5 Hz), 4.61 (1H, d, J=3.5 Hz), 6.87 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz). MS *m/z* (relative intensity): 322 (M^+-58, 0.1), 321 (0.9), 320 (2.7), 275 (1.6), 247 (2.5), 208 (3.0), 199 (15), 137 (18), 121 (100). Exact MS *m/z* Calcd for $C_{19}H_{28}O_4$ (M^+-60): 320.19872. Found: 320.19872. IR ν_{max}^{neat} cm^{-1}: 3400. $[\alpha]_D^{16}$ +97° (c=1.3, $CHCl_3$).

The 3(*S*)-epimer (61 mg, 11%) was next obtained. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7.0 Hz), 1.03 (3H, d, J=7.5 Hz), 1.10 (3H, d, J=6.0 Hz), 1.16 (3H, d, J=5.5 Hz), 1.20—2.25 (7H, m), 3.20—4.05 (5H, m), 3.80 (3H, s), 4.42 (2H, ABq, J=11.0 Hz), 4.53 (1H, s), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0). MS *m/z* (relative intensity): 320 (M^+-60, 5), 274 (2), 247 (6.7), 208 (4), 199 (13), 121 (100). Exact MS *m/z* Calcd for $C_{19}H_{28}O_4$ (M^+-60): 320.19872. Found: 320.19950.

5(*R*)-[2-(*tert*-Butyldimethylsilyloxy)ethyl]-6(*S*)-(2-hydroxy-1(*S*)-methyl-1(*S*)-ethyl)-2(*S*)-isopropyloxy-3(*R*)-methyltetrahydropyran (19)——(a) An ether solution (3 ml) of **18** (39 mg, 0.10 mmol) was hydrogenated in the presence of 5% Rh–Al_2O_3 (18 mg) at 0 °C under ordinary pressure for 5 h. After removal of the catalyst by filtration, the filtrate was evaporated to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 10) to give **19** as a colorless oil (34 mg, 76%). ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.83 (3H, d, J=7.0 Hz), 0.89 (9H, s), 1.00 (3H, d, J=7.5 Hz), 1.15 (3H, d, J=6.5 Hz), 1.22 (3H, d, J=6.5 Hz), 1.15—1.35 (2H, m), 1.50—2.05 (5H, m), 2.74 (1H, dd, J=9.0, 2.5 Hz), 3.55—3.95 (6H, m), 4.64 (1H, d, J=3.0 Hz). MS *m/z* (relative intensity): 374 (M^+, 0.3), 315 (7.1), 297 (5.5), 257 (61), 100 (100). Exact MS *m/z* Calcd for $C_{17}H_{35}O_3Si$ (M^+-59): 315.23550. Found: 315.23511. IR ν_{max}^{neat} cm^{-1}: 3400. $[\alpha]_D^{16}$ +123.5° (c=1.2, $CHCl_3$).

(b) A CH_2Cl_2 solution (100 ml) of **20** (4.3 g, 11.4 mmol), imidazole (1.5 g, 22.4 mmol), and TBDMS chloride (2.1 g, 13.4 mmol) was stirred for 1 h at room temperature. The reaction mixture was washed with brine, dried (Na_2SO_4), and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 20) to afford the TBDMS ether as a colorless oil (5.64 g, 99%). ^{1}H-NMR ($CDCl_3$) δ: 0.03 (6H, s), 0.82 (3H, d, J=7 Hz), 0.88 (9H, s), 0.89 (3H, d, J=7 Hz), 1.07 (3H, d, J=6 Hz), 1.14 (3H, d, J=6 Hz), 1.20—2.20 (6H, m), 3.31 (1H, dd, J=9, 7 Hz), 3.47 (1H, dd, J=9, 7 Hz), 3.60—3.95 (4H, m), 3.79 (3H, s), 4.41 (2H, ABq, J=10.5 Hz), 6.86 (2H, d, J=9 Hz), 7.25 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 435 (M^+-59, 0.1), 434 (0.4), 377 (0.6), 313 (6.2), 181 (5.0), 163 (4.0), 121 (100). Exact MS *m/z* Calcd for $C_{25}H_{42}O_4Si$ (M^+-60): 434.28519. Found: 434.28250.

An EtOAc solution (70 ml) of the TBDMS ether (5.64 g, 11.4 mmol) was hydrogenated over 10% Pd–C (1.0 g) at ordinary temperature and pressure for 2 d. After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 20) to give **19** as a colorless oil (4.05 g, 95%).

(c) DDQ (18 mg, 0.079 mmol) was added to a stirred cold CH_2Cl_2 solution (2 ml) of the above TBDMS ether (23.2 mg, 0.047 mmol) containing H_2O (0.1 ml) in an ice bath. After 1.5 h, the reaction mixture was poured into 10% $NaHCO_3$, and extracted with CH_2Cl_2. The extract was washed with 10% $NaHCO_3$, dried over Na_2SO_4, and evaporated to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 20) to give **19**

as a colorless oil (16.2 mg, 92%).

2(*R*)-5(*R*)-[2-(*tert*-Butyldimethylsilyl)ethyl]-2(*S*)-isopropyloxy-3(*R*)-methyl-6(*S*)-tetrahydropyranylpropanal (21)——A solution of DMSO (180 mg, 2.31 mmol) in CH_2Cl_2 was added dropwise to a stirred CH_2CH_2 solution (5 ml) of $(COCl)_2$ (190 mg, 1.5 mmol) at −80 °C, and then a solution of **19** (277 mg, 0.74 mmol) in CH_2Cl_2 was similarly added. After 1 h, Et_3N (300 mg, 2.97 mmol) was added, and the reaction mixture was allowed to warm gradually to room temperature, then washed with brine, dried over Na_2SO_4, and evaporated *in vacuo* to leave crude **21** as a colorless oil (259 mg, 94%), which was subjected to the next reaction without further purification. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.84 (3H, d, J=7.0 Hz), 0.89 (9H, s), 1.05 (3H, d, J=6.5 Hz), 1.09 (3H, d, J=7.5 Hz), 1.18 (3H, d, J=6.5 Hz), 1.20—1.85 (6H, m), 2.61 (1H, dq, J=1.5, 6.5 Hz), 3.55—3.82 (3H, m), 4.20 (1H, dd, J=10.5, 3.0 Hz), 4.57 (1H, d, J=3.5 Hz), 9.67 (1H, s). IR ν_{max}^{neat} cm^{-1}: 1725.

5(*R*)-[5(*R*)-(2-Hydroxyethyl)-2(*S*)-isopropyloxy-3(*R*)-methyl-6(*S*)-tetrahydropyranyl]-4(*R*)-hydroxy-1-hexane (4)—(a) A stirred 1.4 M ether solution of allylmagnesium bromide (5 ml) was diluted with ether (10 ml) and cooled to −90 °C. Next, a solution of **21** (451 mg, 1.21 mmol) in ether was added dropwise, and after 1.5 h, the reaction mixture was allowed to warm gradually to −5 °C, then quenched by the dropwise addition of cold saturated NH_4Cl solution, and extracted with ether. The extract was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo* to leave a 6.7 : 1 mixture of **22a** and **22b** (450 mg, 90%), which was dissolved in THF (2 ml). To this solution, a 1 M THF solution of *n*-Bu_4NF (1.3 ml) was added. The mixture was stirred overnight at room temperature, and then evaporated *in vacuo*. The residue was chromatographed on a silica gel column with CH_2Cl_2–Et_2O (15 : 1) to give **4** as a colorless oil (286.5 mg, 87%). ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=7.0 Hz), 0.88 (3H, d, J=7.0 Hz), 1.08 (3H, d, J=7.0 Hz), 1.10—1.33 (2H, m), 1.21 (3H, d, J=6.0 Hz), 1.45—1.90 (5H, m), 2.15 (1H, dt, J=15.0, 7.5 Hz), 2.36 (1H, dt, J=15.0, 7.5 Hz), 3.55—4.00 (6H, m), 4.67 (1H, d, J=3.5 Hz), 5.00—5.20 (2H, m), 5.82 (1H, ddt, J=17.0, 10.0, 7.5 Hz). MS *m/z* (relative intensity): 269 (M^+ −31, 0.1), 241 (2.5), 223 (5.0), 199 (8.5), 141 (33), 100 (100). Exact MS *m/z* Calcd for $C_{14}H_{25}O_3$ (M^+ −59): 241.18034. Found: 241.18112. $[\alpha]_D^{16}$ +165.5° (c=0.9, $CHCl_3$).

(b) LAH (37 mg, 1.0 mmol) was added in three portions to a stirred suspension of $CrCl_3$ (300 mg, 1.89 mmol) in THF (10 ml) at 0 °C. The color of the reaction mixture changed from violet to dark brown. Stirring was continued for 5 min at 0 °C and for 20 min at room temperature, then a THF solution (5 ml) of **21** (100 mg, 0.27 mmol) and allyl iodide (136 mg, 0.80 mmol) was added at room temperature with stirring. After 30 min, the reaction mixture was poured into aqueous $NaHCO_3$, and insoluble material was removed by filtration. The filtrate was extracted with CH_2Cl_2. Evaporation of the solvent left a 10 : 1 mixture of **22a** and **22b** (86 mg, 78%), which was converted to **4** as described above.

(4*R*,5*S*,6*S*,7*R*,9*R*)-5,9-Dimethyl-4,6-isopropylidenedioxy-10-(4-nitrobenzoyl)oxy-7-[2-(4-nitrobenzoyl)oxyethyl]-1-decene (23)——DDQ (7.0 mg, 0.031 mmol) was added to a stirred cold solution of **24** (10.7 mg, 0.024 mmol)[2,3] in CH_2Cl_2 (2 ml) and H_2O (0.1 ml) in an ice bath. After 1 h, the reaction mixture was poured into aqueous $NaHCO_3$ solution, and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was subjected to silica gel thin layer chromatography (TLC) with EtOAc–hexane (1 : 2) to give (4*R*,5*S*,6*S*,7*R*,9*R*)-5,9-dimethyl-7-(2-hydroxyethyl)-4,6-isopropylidenedioxy-1-decen-10-ol as an oil (6.3 mg, 88%), which was dissolved in CH_2Cl_2 (0.5 ml). To this stirred solution, Et_3N (0.1 ml), 4-dimethylaminopyridine (DMAP; 5 mg), and 4-nitrobenzoyl chloride (30 mg, 0.16 mmol) were added at room temperature. After 4 h, the reaction mixture was worked up in the usual way to give **23** as a colorless oil (13 mg, quantitative). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=7.0 Hz), 1.05 (3H, d, J=7.0 Hz), 1.10—1.40 (2H, m), 1.33 (3H, s), 1.36 (3H, s), 1.40—2.20 (5H, m), 2.10 (1H, dt, J=15.0, 7.0 Hz), 2.29 (1H, dt, J=15.0, 7.0 Hz), 3.61 (1H, dd, J=9.5, 1.5 Hz), 3.81 (1H, dt, J=2.0, 7.0 Hz), 4.12 (1H, dd, J=10.5, 7.0 Hz), 4.31 (1H, dd, J=10.5, 6.0 Hz), 4.35—4.55 (2H, m), 5.05 (1H, d, J=8.0 Hz), 5.11 (1H, d, J=15.0 Hz), 5.77 (1H, ddt, J=15.0, 8.0, 7.0 Hz), 8.20 (4H, d, J=9.0 Hz), 8.29 (4H, d, J=9.0 Hz). MS *m/z* (relative intensity): 583 (M^+, 14), 523 (2.6), 356 (9.0), 332 (5.6), 292 (5.6), 189 (30), 150 (51), 120 (50), 82 (100). Exact MS *m/z* Calcd for $C_{30}H_{35}N_2O_{10}$ (M^+): 583.22912. Found: 583.22767.

References and Notes

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Highly Stereoselective Total Synthesis of Tylonolide, the Aglycon of the 16-Membered Macrolide Antibiotic Tylosin. II. Total Synthesis of Tylonolide by Virtue of 4-Methoxybenzyl and 3,4-Dimethoxybenzyl Protection[1,2]

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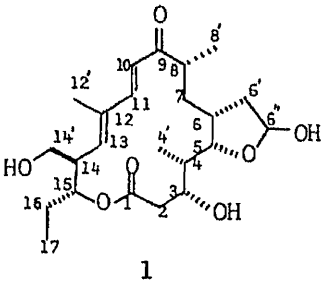
Tylonolide, was synthesized from D-glucose *via* coupling and cyclization of two segments i (**2**) (C-11—C-17) and ii (**3**) (C-1—C-10), which were synthesized from diacetoneglucose. A Prelog–Djerassi lactone-type compound was an intermediate in the synthesis of the latter segment. Esterification of the two segments by Yamaguchi's method followed by macrocyclization by use of the Wittig–Horner reaction gave the 16-membered cyclic enone, whose protecting groups were removed to afford tylonolide. In this total synthesis, 4-methoxybenzyl and 3,4-dimethoxybenzyl protecting groups played an important role.

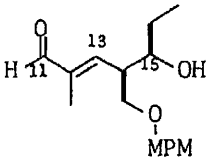
Keywords——macrolide antibiotic; tylosin; aglycon; tylonolide; esterification; macrocyclization; Wittig–Horner reaction; protecting group; 2,3-dichloro-5,6-dicyanobenzoquinone oxidation; stereoselective synthesis

For the purpose of the highly selective total synthesis of complex natural products such as macrolide and polyether antibiotics, it is essential to apply the most suitable protection for many functional groups as well as to use highly regio- and stereo-controlled reactions. As a part of our recent synthetic studies on such complex natural products, we have attempted the highly stereoselective total synthesis of tylonolide (**1**)[3] from D-glucose by means of our new synthetic methodology recently established in the synthesis of methynolide.[4]

Segments i (**2**) and ii (**3**) seemed to be the most suitable intermediate in our general strategy and, in the preceding paper,[1] the synthesis of the chiral synthon (**12**) was reported. In the present paper, we describe a new total synthesis of **1** through syntheses of **2** and **3**, coupling of these two segments, and selective removal of 4-methoxybenzyl (MPM)[5,6] and

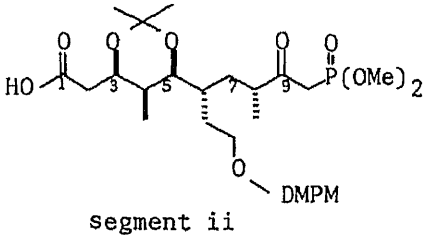


1



segment i

2



segment ii

3

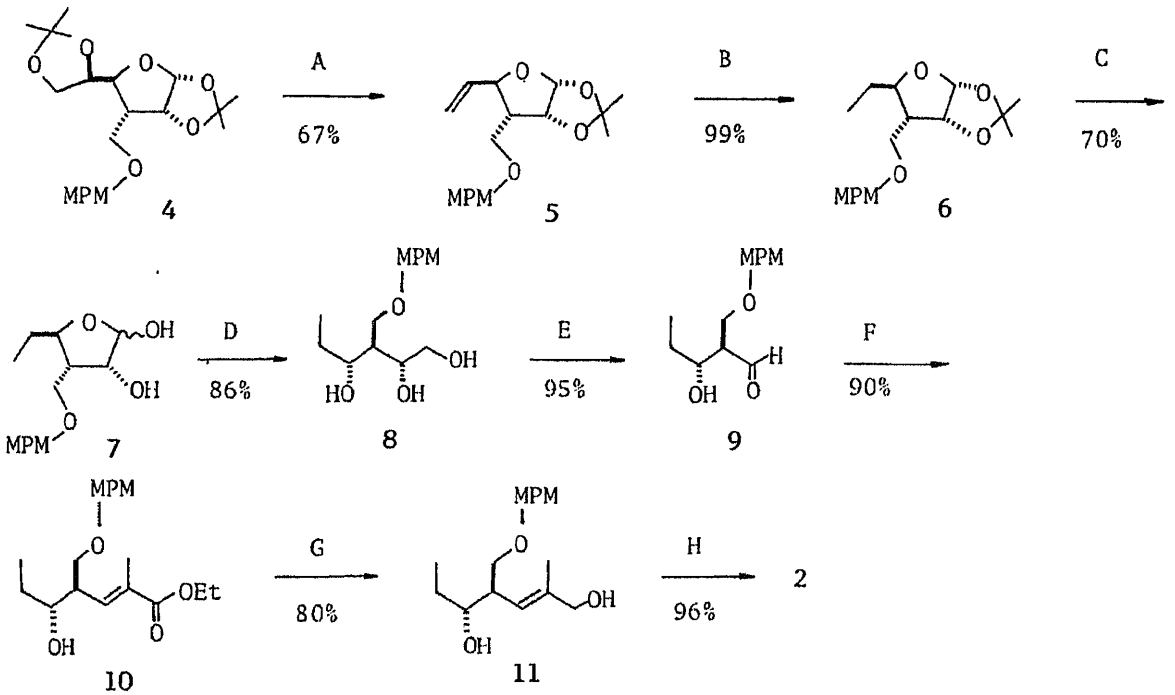
MPM = 4-$MeOC_6H_4CH_2$
DMPM = 3,4-$(MeO)_2C_6H_3CH_2$

Chart 1

3,4-dimethoxybenzyl (DMPM)[6,7] protecting groups by the use of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) at crucial points in the final stage.

Synthesis of Segment i

Segment i (**2**), corresponding to C-11—C-17 of **1**,[8] was rather easily synthesized from D-glucose *via* the MPM ether (**4**).[9,10] The isopropylidene protection of the 5,6-side chain[12] was selectively removed in methanol with 2% sulfuric acid,[13] and the resulting diol was converted to the ditosylate, which was subjected to reductive elimination with sodium iodide in methyl ethyl ketone in the usual way to give the olefin (**5**). Catalytic reduction of **5** over 10% palladium on carbon (Pd–C) gave **6** in quantitative yield. Removal of the 1,2-isopropylidene protection[12] in tetrahydrofuran (THF) with 4.5 N hydrochloric acid gave the lactol (**7**), which was reduced to the triol (**8**) by treatment with calcium borohydride[14] in high yield.[15] The 1,2-diol was readily cleaved with sodium metaperiodate to give the aldehyde (**9**) in excellent yield. When **9** was treated with a stable ylide[16] in benzene at 60 °C, a smooth Wittig reaction proceeded, and the expected (*E*)-α,β-unsaturated ester (**10**) was isolated in high yield.[17,18] Lithium aluminum hydride (LAH) reduction of the ester (**10**) gave the allyl alcohol (**11**), which was oxidized with manganese dioxide in dichloromethane to give the α,β-unsaturated aldehyde (**2**) (segment i) in excellent yield.



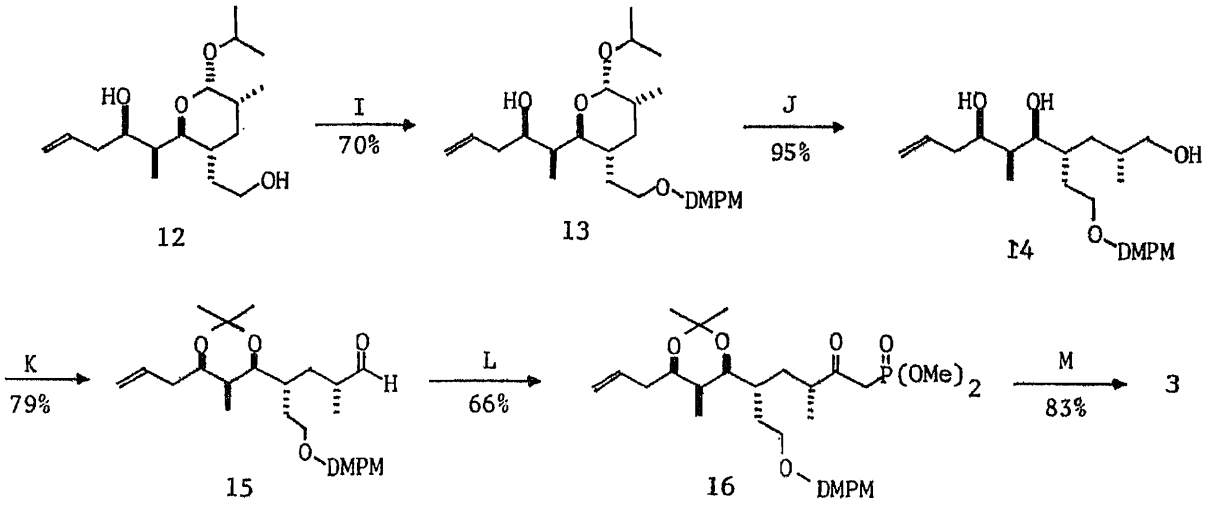
(A) 1) 2% H_2SO_4, MeOH; 2) TsCl, Et_3N, DMAP, CH_2Cl_2; 3) NaI, MeCOEt, reflux (B) Pd–C, H_2, EtOAc (C) 4.5 N HCl, THF, 50 °C (D) $CaCl_2$, $NaBH_4$, EtOH (E) $NaIO_4$, MeOH–H_2O (F) $Ph_3P{=}CMeCO_2Et$, benzene, 60 °C (G) $LiAlH_4$, THF, 0 °C (H) MnO_2, CH_2Cl_2

Chart 2

Synthesis of Segment ii

In the previous paper,[1] the chiral intermediate (**12**) having all the chiral centers required for segment ii was synthesized from D-glucose. The primary alcohol of **12** was protected with a DMPM group[6,7] to give **13** in 70% yield,[19] and then the acetal of **13** was hydrolyzed in THF with 1 N hydrochloric acid. The resulting lactol was reduced to the triol (**14**) by treatment with calcium borohydride[14] in quantitative yield.[20] The 1,3-diol group of **14** was protected as an

acetonide group with 2,2-dimethoxypropane in the presence of camphorsulfonic acid (CSA) in 85% yield, and the remaining primary alcohol was subjected to Swern oxidation[21] to give the aldehyde (**15**) in 98% yield. The Wittig–Horner reaction of **15** with the lithio derivative of dimethyl methylphosphonate[22] gave almost quantitatively a hydroxy phosphonate, which was immediately oxidized with pyridinium dichromate (PDC)[23] in dimethylformamide (DMF) to give the ketophosphonate (**16**).[24] Finally, among various possible oxidation methods of the terminal olefin of **16** into the carboxylic group, Lemieux-von Rudloff oxidation with potassium permanganate and sodium periodate[26] gave a good result, and the expected segment ii (**3**) was isolated in 83% yield.



(I) NaH, DMSO–THF, DMPMCl (J) 1) 1 N HCl, THF, 50 °C; 2) $CaCl_2$, $NaBH_4$, EtOH (K) 1) $(MeO)_2CMe_2$, CSA, benzene; 2) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −80 °C→room temperature (L) 1) $(MeO)_2P(O)Me$, *n*-BuLi, THF, −70→−30 °C; 2) PDC, DMF (M) $NaIO_4$, $KMnO_4$, $NaHCO_3$, $MeCOMe-H_2O$

Chart 3

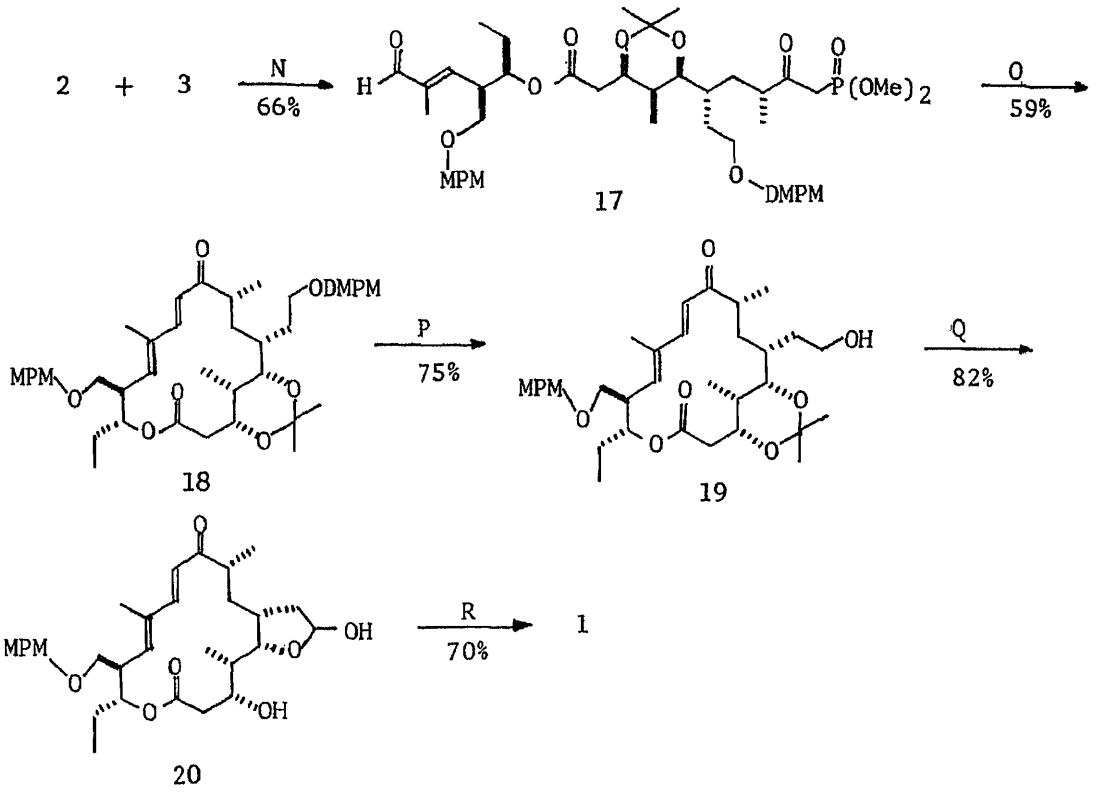
Coupling of the Two Segments and Synthesis of Tylonolide

The macrolactonization of seco-acids is commonly used for the synthesis of macrolides.[27] Wittig–Horner coupling between the two segments i (**2**) and ii (**3**) was first examined in order to obtain a seco-acid, but all attempts were unsuccessful. Therefore, we decided to reverse the order of reactions, namely esterification between **2** and **3** was first examined, and the resulting ester was subjected to intramolecular Wittig–Horner reaction.

When **2** and **3** were coupled in a rather concentrated solution (more than 1 M) with dicyclohexylcarbodiimide (DCC) in the presence of 4-dimethylaminopyridine (DMAP), the expected ester (**17**) was obtained in 61% yield, but unfortunately complete separation from impurities could not be achieved. However, the Yamaguchi method[27g] gave a much better result. When **2** and **3** were condensed with 2,4,6-trichlorobenzoyl chloride in the presence of triethylamine and DMAP, the esterification was completed within only 1 h at room temperature to give the ester (**17**) in 66% yield.

The intramolecular Wittig–Horner coupling of **17** was carried out under Aristoff[28]–Nicolaou[3b] conditions, namely when a 1 mM solution of **17** in toluene was heated at 100 °C in the presence of 18-crown-6 (12 eq) and potassium carbonate (6 eq), the starting material (**17**) disappeared after 12 h, and the expected 16-membered enone (**18**) was isolated in 59% yield.

Selective removal of DMPM protecting groups in the presence of MPM protecting groups by DDQ oxidation has been clearly demonstrated in many cases.[6,7] When an ice-cold



(N) 2,4,6-$Cl_3C_6H_2COCl$, Et_3N, DMAP, THF (O) K_2CO_3, 18-crown-6, toluene, 100 °C (P) DDQ, CH_2Cl_2–H_2O–benzene, 5 °C (Q) 1) PDC, CH_2Cl_2; 2) 0.5 N HCl, THF (R) DDQ, CH_2Cl_2–H_2O

Chart 4

solution of **18** in dichloromethane–water (20 : 1) was treated with a slight excess of DDQ (1.2 eq), the selective deprotection of the DMPM group occurred smoothly, and the monoalcohol (**19**) was isolated in 75% yield. PDC oxidation[23] of the primary alcohol of **19** gave the aldehyde (94%), and the remaining acetonide was immediately removed with 0.5 N hydrochloric acid to give the hemiacetal compound (**20**) in 90% yield. Finally, the MPM protecting group of **20** was easily removed by retreatment with DDQ under usual conditions,[5,6] and tylonolide (**1**) was isolated in 70% yield. This compound was identical, in terms of its infrared (IR), nuclear magnetic resonance (NMR) and mass spectra (MS) with tylonolide derived from natural tylosin.[29,30] Stereoselectivities in this total synthesis of **1** from D-glucose for the construction of the new chiral centers at C-3, C-4, C-6, C-8, and C-14 were 91, 94, 100, 87, and 100%, respectively.

Experimental

Physical data were measured as described in the previous paper.[4a]

1,2-*O*-Isopropylidene-3-*C*-(4-methoxybenzyl)oxymethyl-3,5,6-trideoxy-α-D-*ribo*-hex-5-enofuranose (5)——A solution of **4** (3.52 g, 8.95 mmol) in MeOH (43 ml) and 2% H_2SO_4 (18 ml) was allowed to stand at room temperature for 15 h. After neutralization with $NaHCO_3$, the reaction mixture was evaporated *in vacuo*, and extracted with CH_2Cl_2. The extract was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (2 : 1) to give the diol as a colorless oil (2.72 g, 86.5%). TsCl (1.68 g), Et_3N (2 g) and DMAP (50 mg) were added to a stirred CH_2Cl_2 solution (50 ml) of the diol (1.31 g, 3.8 mmol) at room temperature. After 19 h, the reaction mixture was diluted with CH_2Cl_2, washed with 1 N HCl, 10% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave a pale yellow oil, which was dissolved in MeCOEt and heated under reflux with NaI (3.42 g) for 24 h. The reaction mixture was evaporated *in*

vacuo to leave an oil, which was extracted with CH_2Cl_2. The extract was washed with $Na_2S_2O_3$ solution and H_2O, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 3) to give **5** as a colorless solid (0.91 g, 77%). Recrystallization from ether–hexane gave colorless needles, mp 54—56 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, s), 1.51 (3H, s), 1.94—2.23 (1H, m), 3.40 (1H, dd, J=9.5, 5.0 Hz), 3.75 (1H, dd, J=9.5, 9.0 Hz), 3.80 (3H, s), 4.20 (1H, dd, J=10.5, 9.0 Hz), 4.46 (2H, s), 4.74 (1H, t, J=4.0 Hz), 5.15—5.39 (2H, m), 5.65—6.00 (1H, m), 5.83 (1H, d, J=4.5 Hz), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz). MS *m/z* (relative intensity): 320 (M^+, 0.2), 305 (0.3), 262 (3.5), 137 (41), 121 (100). Exact MS *m/z* Calcd for $C_{18}H_{24}O_5$: 320.1623. Found: 320.16241. *Anal.* Calcd for $C_{18}H_{24}O_5$: C, 67.48; H, 7.55. Found: C, 67.33; H, 7.55.

1,2-*O*-Isopropylidene-3-*C*-(4-methoxybenzyl)oxymethyl-3,5,6-trideoxy-α-D-*ribo*-hexofuranose (6)——An EtOAc solution (30 ml) of **5** (1.38 g, 4.31 mmol) was hydrogenated with 10% Pd–C (100 mg) at ordinary temperature and pressure for 30 min. After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* to leave **6** as a colorless oil (1.36 g, 98.5%). ^{1}H-NMR ($CDCl_3$) δ: 0.98 (3H, t, J=7.0 Hz), 1.20—2.15 (3H, m), 1.32 (3H, s), 1.49 (3H, s), 3.44 (1H, dd, J=9.5, 6.0 Hz), 3.65—3.91 (2H, m), 3.81 (3H, s), 4.47 (2H, s), 4.70 (1H, t, J=4.0 Hz), 5.79 (1H, d, J=3.5 Hz), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz). $[\alpha]_D^{15}+50°$ (c=1.3, $CHCl_3$). MS *m/z* (relative intensity): 307 (M^+-15, 0.4), 264 (2.9), 203 (1.0), 138 (58), 121 (100). Exact MS *m/z* Calcd for $C_{17}H_{23}O_5$ (M^+-15): 307.15452. Found: 307.15520.

3-*C*-(4-Methoxybenzyl)oxymethyl-3,5,6-trideoxy-α-D-*ribo*-hexofuranose (7)——A solution of **6** (1.63 g, 5.06 mmol) in THF (30 ml) and 4.5 N HCl (10 ml) was heated at 50 °C for 5.5 h. After neutralization with $NaHCO_3$, the reaction mixture was evaporated *in vacuo*, and the residue was extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*, and the residue was chromatographed on a silica gel column with EtOAc–hexane (2 : 1) to give **7** (1.0 g, 70%). Recrystallization from ether–hexane gave colorless crystals, mp 61—64 °C. MS *m/z* (relative intensity): 282 (M^+, 0.8), 264 (1.2), 203 (1.2), 137 (43), 121 (100). Exact MS *m/z* Calcd for $C_{15}H_{22}O_5$ (M^+): 282.14669. Found: 282.14862. *Anal.* Calcd for $C_{15}H_{22}O_5$: C, 63.80; H, 7.85. Found: C, 63.66; H, 7.81.

(2*R*,3*R*,4*S*)-2-(4-Methoxybenzyl)oxymethyl-1,2,4-trihydroxyhexane (8)——A solution of $NaBH_4$ (400 mg, 10.5 mmol) in EtOH (50 ml) was added dropwise to a cold stirred EtOH solution (50 ml) of $CaCl_2$ (1.1 g) at −20 °C under argon. The resulting $Ca(BH_4)_2$ solution was stirred for 30 min below −10 °C, then a solution of **7** (900 mg, 3.19 mmol) in EtOH was added and the mixture was stirred at room temperature for 1 h. The reducing agent was decomposed with 4 N HCl, and the solution was neutralized with $NaHCO_3$. After removal of precipitates by filtration, the filtrate was evaporated *in vacuo* and the residue was extracted with CH_2Cl_2. The extract was dried and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–EtOH (20 : 1) to give **8** as a colorless oil (777 mg, 86%). ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, t, J=7 Hz), 1.30—1.70 (2H, m), 1.88 (1H, dq, J=4, 6 Hz), 3.52 (1H, dd, J=5, 9.5 Hz), 3.58 (1H, dd, J=3.5, 9.5 Hz), 3.65 (1H, dd, J=5, 11.5 Hz), 3.72 (1H, dd, J=4, 11.5 Hz), 3.75—3.88 (1H, m), 3.81 (3H, s), 3.98 (1H, dt, J=4.5, 5 Hz), 4.41 (2H, s), 6.88 (2H, d, J=9 Hz), 7.22 (2H, d, J=9 Hz). MS *m/z* (relative intensity): 284 (M^+, 0.5), 266 (3.8), 178 (1.8), 150 (2.6), 137 (44), 121 (100). Exact MS *m/z* Calcd for $C_{15}H_{24}O_5$ (M^+): 284.16235. Found: 284.16259. $[\alpha]_D^{15}+7.8°$ (c=1.3, $CHCl_3$).

(2*S*,3*R*)-3-Hydroxy-2-[(4-methoxybenzyl)oxymethyl]pentanal (9)——A solution of $NaIO_4$ (430 mg, 2.0 mmol) in H_2O (3.5 ml) was added to a stirred MeOH solution (10 ml) of **8** (194 mg, 0.68 mmol) at room temperature. After 1 h, the precipitate was filtered off, and the filtrate was evaporated *in vacuo*. The residue was taken up in CH_2Cl_2, and the solution was dried over Na_2SO_4, then evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 1) to give **9** as a colorless oil (164 mg, 95%). ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, t, J=7.0 Hz), 1.60 (2H, quint, J=7.0 Hz), 2.45—2.63 (1H, m), 2.80 (1H, br s), 3.67—4.20 (3H, m), 3.81 (3H, s), 4.46 (2H, s), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz), 9.87 (1H, d, J=1.5 Hz). MS *m/z* (relative intensity): 252 (M^+, 0.4), 234 (0.4), 194 (1.8), 175 (10), 137 (92), 121 (100). Exact MS *m/z* Calcd for $C_{14}H_{20}O_4$ (M^+): 252.13614. Found: 252.13501. IR ν_{max}^{neat} cm^{-1}: 3400, 1720. $[\alpha]_D^{15}+6.9°$ (c=1.3, $CHCl_3$).

Ethyl 5(*R*)-Hydroxy-4(*R*)-(4-methoxybenzyl)oxymethyl-2-methyl-2(*E*)-heptenoate (10)——A benzene solution (20 ml) of **9** (545 mg, 2.16 mmol) and recrystallized 1-carboethoxyethylidenetriphenylphosphorane[16] (1.6 g, 4.4 mmol) was heated at 60 °C for 10 h. After evaporation of the solvent *in vacuo*, the residue was chromatographed on a silica gel column with EtOAc–hexane (1 : 2) to give **10** as a colorless oil (653 mg, 90%). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=7.0 Hz), 1.30 (3H, t, J=7.0 Hz), 1.25—1.60 (2H, m), 1.86 (3H, d, J=1.5 Hz), 2.50 (1H, br s), 2.60—2.90 (1H, m), 3.53 (1H, dd, J=12.0, 1.0 Hz), 3.63 (1H, dd, J=12.0, 2.5 Hz), 3.70—3.95 (1H, m), 3.81 (3H, s), 4.19 (2H, q, J=7.0 Hz), 4.45 (2H, s), 6.81 (1H, dq, J=10.0, 1.5 Hz), 6.86 (2H, d, J=9.0 Hz), 7.25 (2H, d, J=9.0 Hz). IR ν_{max}^{neat} cm^{-1}: 3450, 1705. $[\alpha]_D^{16.5}-17°$ (c=4.4, $CHCl_3$). MS *m/z* (relative intensity): 336 (M^+, 0.4), 263 (0.8), 200 (10), 121 (100). Exact MS *m/z* Calcd for $C_{19}H_{28}O_5$ (M^+): 336.19364. Found: 336.19474.

1,5(*R*)-Dihydroxy-4(*R*)-(4-methoxybenzyl)oxymethyl-2-methyl-2(*E*)-heptene (11)——A THF solution of **10** (454 mg, 1.35 mmol) was added dropwise to a cold stirred suspension of $LiAlH_4$ (100 mg, 2.6 mmol) in THF (5 ml) under argon in an ice-bath. After 1 h, the reaction was quenched by the addition of MeOH, then H_2O (0.1 ml), 15% NaOH (0.1 ml) and H_2O (0.3 ml) were added, and the mixture was stirred for 1 h. After removal of precipitated inorganic salts by filtration, the filtrate was evaporated *in vacuo* and the residue was chromatographed on a silica gel column with EtOAc–hexane (2 : 1) to give **11** as a colorless solid (339 mg, 86%). Recrystallization from benzene–

hexane gave colorless needles, mp 78—80 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=7.5 Hz), 1.42 (2H, quint, J=7.5 Hz), 1.69 (3H, d, J=1.5 Hz), 1.78 (2H, br s), 2.60—2.75 (1H, m), 3.44—3.62 (2H, m), 3.72 (1H, dt, J=3.0, 7.5 Hz), 3.81 (3H, s), 4.03 (2H, d, J=1.0 Hz), 4.50 (1H, dq, J=10.5, 1.5 Hz), 6.86 (2H, d, J=9.0 Hz), 7.24 (2H, d, J=9.0 Hz). MS m/z (relative intensity): 294 (M^+, 1.4), 155 (2.8), 138 (22), 121 (100). Exact MS m/z Calcd for $C_{17}H_{26}O_4$ (M^+): 294.18307. Found: 294.18409. *Anal.* Calcd for $C_{17}H_{26}O_4$: C, 69.36; H, 8.90. Found: C, 69.22; H, 9.00.

5(*R*)-Hydroxy-4(*R*)-(4-methoxybenzyl)oxymethyl-2-methyl-2(*E*)-heptenal (2)——A CH_2Cl_2 solution (2 ml) of **11** (17.0 mg, 0.058 mmol) was stirred with active MnO_2 (100 mg, 1.15 mmol) at room temperature for 1 h. After removal of the precipitate by filtration, the filtrate was evaporated *in vacuo* and chromatographed on a silica gel column with EtOAc–hexane (1 : 3) to leave **2** as a colorless oil (16.2 mg, 96%). ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=7.0 Hz), 1.20—1.60 (2H, m), 1.75 (3H, d, J=1.0 Hz), 2.7 (1H, br s), 2.89 (1H, ddt, J=10.0, 2.0, 5.0 Hz), 3.64 (2H, d, J=5.0 Hz), 3.80 (3H, s), 3.80—4.00 (1H, m), 4.45 (2H, s), 6.67 (1H, dd, J=10.0, 1.0 Hz), 6.86 (2H, d, J=8.5 Hz), 7.21 (2H, d, J=8.5 Hz), 9.41 (1H, s). IR ν_{max}^{neat} cm^{-1}: 3430, 1680.

5(*S*)-{5(*R*)-[2-(3,4-Dimethoxybenzyloxy)ethyl]-2(*S*)-isopropyloxy-3(*R*)-methyl-6(*S*)-tetrahydropyranyl}-4(*R*)-hydroxy-1-hexene (13)——A solution of **12** (144 mg, 0.48 mmol) in THF (2 ml) was added dropwise to a stirred suspension of NaH (100 mg, 2.5 mmol) in dimethyl sulfoxide (DMSO) (2 ml) at room temperature. After 1 h, DMPM chloride (93.3 mg, 0.50 mmol) was added, and stirring was continued for 10 h. Then Et_3N (1 ml) was added, and after 30 min, the reaction mixture was poured into saturated NH_4Cl solution and extracted with CH_2Cl_2. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 5) to give **13** as a colorless oil (151.2 mg, 70%). ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, d, J=7 Hz), 0.91 (3H, d, J=7 Hz), 1.09 (3H, d, J=6 Hz), 1.22 (3H, d, J=6 Hz), 1.15—1.35 (1H, m), 1.47 (1H, dt, J=12.5, 4 Hz), 1.62 (1H, br s), 1.57—1.90 (4H, m), 2.14 (1H, dt, J=13.5, 7 Hz), 2.34 (1H, dt, J=13.5, 7 Hz), 3.40—3.55 (2H, m), 3.73 (1H, dd, J=2, 10.5 Hz), 3.73 (1H, d, J=8 Hz), 3.87 (1H, sept, J=6 Hz), 3.88 (3H, s), 3.89 (3H, s), 4.40 (1H, d, J=12 Hz), 4.46 (1H, d, J=12 Hz), 4.67 (1H, d, J=3 Hz), 5.03 (1H, d, J=10 Hz), 5.08 (1H, d, J=17 Hz), 5.79 (1H, ddt, J=10, 17, 7 Hz), 6.80—6.90 (3H, m). MS m/z (relative intensity): 450 (M^+, 1.0), 390 (3.1), 319 (1.5), 239 (2.2), 222 (19), 151 (100). Exact MS m/z Calcd for $C_{26}H_{42}O_6$ (M^+): 450.29809. Found: 450.29840. $[\alpha]_D^{16}$ +119° (c=0.9, $CHCl_3$).

Monoacetate (colorless oil). ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=6.5 Hz), 0.88 (3H, d, J=7.0 Hz), 1.08 (3H, d, J=6.5 Hz), 1.19 (3H, d, J=6.5 Hz), 1.20—2.10 (7H, m), 2.03 (3H, s), 2.27 (1H, dt, J=15.5, 7.5 Hz), 2.45—2.64 (1H, m), 3.46 (2H, t, J=6.5 Hz), 3.62 (1H, dd, J=5.0, 1.0 Hz), 3.80—4.05 (1H, m), 3.88 (3H, s), 3.89 (3H, s), 4.39 (1H, d, J=11.0 Hz), 4.45 (1H, d, J=11.0 Hz), 4.62 (1H, d, J=3.0 Hz), 4.95—5.10 (3H, m), 5.63—5.80 (1H, m), 6.83—6.90 (3H, m).

(4*R*,5*S*,6*S*,7*R*,9*R*)-7-[2-(3,4-Dimethoxybenzyloxy)ethyl]-5,9-dimethyl-4,6,10-trihydroxy-1-decene (14)——A solution of **13** (1.97 g, 4.37 mmol) in THF (50 ml) and 1 N HCl (15 ml) was heated at 50 °C for 2 h. After neutralization with $NaHCO_3$, the reaction mixture was evaporated *in vacuo* and extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 1) to give the lactol as a colorless oil (1.70 g, 95%).

A solution of $NaBH_4$ (1.5 g, 40 mmol) in EtOH (100 ml) was added dropwise to a stirred cold EtOH solution (200 ml) of $CaCl_2$ (3.0 g, 27 mmol) at −10 °C. After 30 min, the above lactol (1.70 g, 4.16 mmol) in EtOH (50 ml) was added, and the stirred reaction mixture was allowed to warm to room temperature. After 1.5 h, 1 N HCl was added to decompose the reducing agent, and the mixture was neutralized with $NaHCO_3$. After removal of precipitated inorganic salts by filtration, the filtrate was evaporated *in vacuo* and extracted with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated *in vacuo* to leave an oil, which was dissolved in MeOH containing AcOH (0.5 ml) to decompose borates. After evaporation of the solvent *in vacuo*, the acidic residue was neutralized with $NaHCO_3$ and then extracted with CH_2Cl_2. The extract was dried and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column. Elution with EtOAc and then CH_2Cl_2–MeOH (10 : 1) gave **14** as a colorless oil (1.7 g, 100%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (6H, d, J=7.0 Hz), 1.10—1.90 (7H, m), 2.05—2.42 (2H, m), 3.20—3.95 (9H, m), 3.88 (3H, s), 3.89 (3H, s), 4.42 (2H, s), 5.03—5.19 (2H, m), 5.29—5.90 (1H, m), 6.84—6.90 (3H, m). $[\alpha]_D^{16}$ +2.3° (c=1.6, $CHCl_3$). MS m/z (relative intensity): 410 (M^+, 1.5), 392 (1), 310 (1.5), 292 (1.5), 282 (2), 151 (100). Exact MS m/z Calcd for $C_{23}H_{38}O_6$ (M^+): 410.26679. Found: 410.26802.

(2*R*,4*R*,5*S*,6*S*,7*R*)-4-[2-(3,4-Dimethoxybenzyloxy)ethyl]-2,6-dimethyl-5,7-isopropylidenedioxy-9-decenal (15)——A benzene solution (50 ml) of **14** (1.70 g, 4.14 mmol), 2,2-dimethoxypropane (5 ml) and CSA (20 mg) was stirred at room temperature for 3 h. The reaction mixture was washed with aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 3) to give an oil. The oil was dissolved in CH_2Cl_2 and the solution was shaken with 1 N HCl. Evaporation of the solvent gave (4*R*,5*S*,6*S*,7*R*,9*R*)-7-[2-(3,4-dimethoxybenzyloxy)ethyl]-5,9-dimethyl-4,6-isopropylidenedioxy-1-decen-10-ol as a colorless oil (1.56 g, 84%). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, d, J=6.5 Hz), 0.85 (3H, d, J=6.5 Hz), 1.05—2.00 (8H, m), 1.41 (3H, s), 1.42 (3H, s), 2.01—2.42 (2H, m), 3.20—3.95 (6H, m), 3.88 (3H, s), 3.89 (3H, s), 4.43 (2H, s), 5.05 (1H, br d, J=10.0 Hz), 5.08 (1H, br d, J=17.0 Hz), 5.53—5.79 (1H, m), 6.84—6.88 (3H, m). MS m/z (relative intensity): 450 (M^+, 1.5), 398 (1.5), 310 (3.5), 204 (3.1), 151 (100). Exact MS m/z Calcd for $C_{26}H_{42}O_6$ (M^+): 450.29809. Found: 450.29810. $[\alpha]_D^{15}$ + 16° (c=2.0, $CHCl_3$).

A solution of DMSO (95 mg, 1.2 mmol) in CH_2Cl_2 (1 ml) was added to a cold stirred CH_2Cl_2 solution of $(COCl)_2$ (77 mg, 0.6 mmol) at −80 °C, and then the above alcohol (182 mg, 0.40 mmol) in CH_2Cl_2 was similarly added. After 1 h, Et_3N (134 mg, 1.3 mmol) was added, then the reaction mixture was allowed to warm to room temperature, washed with H_2O, 0.5 N HCl, brine and aqueous $NaHCO_3$, dried over Na_2SO_4, and evaporated to leave **15** as an oil (172 mg, 94%). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=7.0 Hz), 1.04 (3H, d, J=7.0 Hz), 1.10—2.70 (9H, m), 1.35 (3H, s), 1.38 (3H, s), 3.41—3.95 (4H, m), 3.88 (3H, s), 3.89 (3H, s), 4.42 (2H, s), 5.04 (1H, br d, J=10.0 Hz), 5.08 (1H, br d, J=17.0 Hz), 5.53—5.87 (1H, m), 6.84—6.88 (3H, m), 9.55 (1H, d, J=2.0 Hz). IR ν_{max}^{neat} cm^{-1}: 1720.

Dimethyl (3*R*,5*R*,6*S*,7*S*,8*R*)-5-[2-(3,4-Dimethoxybenzyloxy)ethyl]-3,7-dimethyl-6,8-isopropylidenedioxy-2-oxo-10-undecenylphosphonate (16)——A 1.6 M BuLi hexane solution (440 μl) was added to a stirred THF solution (6 ml) of dimethyl methylphosphonate (120 μl, 1.0 mmol) at −70 °C under argon. After 30 min, **15** (172 mg, 0.40 mmol) in THF was added dropwise, then the reaction mixture was allowed to warm to −30 °C, quenched by the addition of saturated NH_4Cl, and extracted with ether. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to leave the hydroxyphosphonate as a colorless oil (215 mg, 98%), which was dissolved in DMF (6.5 ml). PDC (500 mg) was added to the resulting solution, and the mixture was stirred for 1 d. Further PDC (500 mg) was added and stirring was continued for 5 h. The reaction mixture was poured into H_2O and extracted with ether. The extract was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (2 : 1) to give **16** as a colorless oil (146 mg, 67%). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, d, J=7.0 Hz), 1.06 (3H, d, J=7.0 Hz), 1.10—1.90 (6H, m), 1.40 (3H, s), 1.41 (3H, s), 2.11 (1H, dt, J=15.0, 7.0 Hz), 2.29 (1H, dt, J=15.0, 7.0 Hz), 2.84—3.04 (1H, m), 3.15 (2H, d, J=22.0 Hz), 3.45—3.90 (4H, m), 3.74 (3H, d, J=1.0 Hz), 3.80 (3H, d, J=10.0 Hz), 3.88 (3H, m), 3.89 (3H, s), 4.41 (2H, s), 5.04 (1H, d, J=9.0 Hz), 5.10 (1H, d, J=15.0 Hz), 5.50—5.90 (1H, m), 6.82—6.89 (3H, m). IR ν_{max}^{neat} cm^{-1}: 1715. MS m/z (relative intensity): 570 (M^+, 0.7), 442 (0.5), 232 (12), 193 (12), 180 (23), 151 (100). Exact MS m/z Calcd for $C_{29}H_{47}O_9P$ (M^+): 570.29571. Found: 570.29603.

(3*R*,4*S*,5*S*,6*R*,8*R*)-6-[2-(3,4-Dimethoxybenzyloxy)ethyl]-10-(dimethoxyphosphono)-4,8-dimethyl-3,5-isopropylidenedioxy-9-oxodecanoic Acid (3)——A solution of **16** (95 mg, 0.16 mmol) in acetone, 10% aqueous $NaHCO_3$ (0.2 ml) and a solution of $KMnO_4$ (5 mg, 0.03 mmol) in H_2O (0.2 ml) were added successively to a stirred aqueous solution (5 ml) of $NaIO_4$ (340 mg, 1.6 mmol) at room temperature. After 5 h, the reaction mixture was filtered, then the filtrate was mixed with saturated NH_4Cl solution (10 ml), and the mixture was extracted with CH_2Cl_2. The extract was washed with aqueous NH_4Cl and brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave **3** as a colorless viscous oil (82 mg, 83%). ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, d, J=7.0 Hz), 1.06 (3H, d, J=7.0 Hz), 1.10—2.00 (6H, m), 1.39 (3H, s), 1.44 (3H, s), 2.37 (1H, dd, J=16.0, 5.0 Hz), 2.58 (1H, dd, J=16.0, 8.0 Hz), 2.80—3.20 (1H, m), 3.14 (2H, d, J=22.0 Hz), 3.47 (2H, t, J=6.0 Hz), 3.72 (3H, d, J=1.0 Hz), 3.83 (3H, d, J=1.0 Hz), 3.85—4.00 (1H, m), 3.88 (3H, s), 3.89 (3H, s), 4.20—4.40 (1H, m), 4.41 (2H, s), 6.85 (3H, s). IR ν_{max}^{neat} cm^{-1}: 1720, 1710. $[\alpha]_D^{26.5}$ −5.6° (c=1.4, $CHCl_3$).

1(*R*)-Ethyl-5-formyl-2(*R*)-(4-methoxybenzyl)oxymethyl-3(*Z*)-pentenyl (3*R*,4*S*,5*S*,6*R*,8*R*)-6-[2-(3,4-Dimethoxybenzyl)oxyethyl]-10-(dimethoxyphosphono)-4,8-dimethyl-3,5-isopropylidenedioxy-9-oxodecanoate (17)——2,4,6-Trichlorobenzoyl chloride (10 μl, 0.06 mmol) was added dropwise to a stirred THF solution (0.5 ml) of **3** (35 mg, 0.059 mmol) and Et_3N (10 μl, 0.07 mmol) at room temperature under argon. After 20 min, the precipitates were filtered off, and the filtrate was evaporated *in vacuo*. The residue was dissolved in benzene (1 ml) and added dropwise to a stirred benzene solution (0.5 ml) of **2** (25 mg, 0.086 mmol) and DMAP (9 mg, 0.07 mmol) at room temperature. After 1 h, the reaction mixture was washed with brine and 10% $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and evaporated to leave an oil, which was subjected to silica gel preparative thin layer chromatography (TLC) with EtOAc–hexane (2 : 1) to give **17** as a colorless oil (34.1 mg, 66%). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, d, J=7.0 Hz), 0.86 (3H, t, J=7.5 Hz), 1.06 (3H, d, J=7.0 Hz), 1.10—2.00 (8H, m), 1.36 (3H, s), 1.41 (3H, s), 1.78 (3H, s), 2.21 (1H, dd, J=15.0, 4.0 Hz), 2.51 (1H, dd, J=15.0, 9.0 Hz), 2.85—3.25 (2H, m), 3.13 (2H, d, J=22.0 Hz), 3.38—3.60 (4H, m), 3.63—4.05 (2H, m), 3.74 (3H, d, J=1.0 Hz), 3.80 (6H, s), 3.87 (3H, s), 3.88 (3H, s), 4.32—4.45 (4H, m), 5.21 (1H, m), 6.45 (1H, d, J=10.0 Hz), 6.80—6.95 (5H, m), 7.20 (2H, d, J=9.0 Hz), 9.44 (1H, s). MS m/z (relative intensity): 741 (M^+ −121, 0.9), 683 (1.5), 363 (2.0), 345 (3.3), 263 (10), 232 (15), 180 (22), 151 (100). Exact MS m/z Calcd for $C_{34}H_{52}O_{12}P$ (M^+ −179): 683.31956. Found: 683.32219. IR ν_{max}^{neat} cm^{-1}: 1735, 1710, 1690. $[\alpha]_D^{26}$ +2.8° (c=1.3, $CHCl_3$).

6″-Dihydro-6″-*O*-(3,4-dimethoxybenzyl)-3,5-*O*-isopropylidene-14′-*O*-(4-methoxybenzyl)tylonolide (18)——A toluene solution (5 ml) of **17** (34.1 mg, 0.039 mmol) was added very slowly to a stirred suspension of K_2CO_3 (33 mg, 0.24 mmol) in toluene (50 ml) in the presence of 18-crown-6 (125 mg, 0.47 mmol) at 100 °C during 1 h, and stirring was continued at the same temperature for 20 h. After cooling to room temperature, the reaction mixture was mixed with saturated NH_4Cl solution and extracted with ether. The extract was washed with saturated KCl solution several times, dried over Na_2SO_4, and evaporated to leave an oil, which was purified by silica gel preparative TLC with EtOAc–hexane (1 : 2) to give **18** as a colorless oil (17.2 mg, 59%). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=6.5 Hz), 0.88 (3H, t, J=7.0 Hz), 1.10—2.20 (8H, m), 1.13 (3H, d, J=6.5 Hz), 1.38 (3H, s), 1.43 (3H, s), 1.82 (3H, s), 2.34 (2H, t, J=5.0 Hz), 2.80—3.10 (3H, m), 3.40 (2H, t, J=6.5 Hz), 3.46 (2H, d, J=5.0 Hz), 3.81 (3H, s), 3.90 (3H, s), 3.90—4.20 (2H, m), 4.35—4.50 (4H, m), 4.99 (1H, dt, J=4.0, 7.5 Hz), 5.81 (1H, d, J=11.0 Hz), 6.17 (1H, d, J=16.0 Hz), 6.80—6.95 (5H, m), 7.17 (1H, d, J=16.0 Hz), 7.20 (2H, d, J=9.0 Hz). MS m/z (relative intensity): 736 (M^+, 0.55), 600 (0.5),

585 (0.9), 512 (0.95), 376 (3.0), 165 (13), 151 (87), 121 (100). Exact MS *m/z* Calcd for $C_{34}H_{49}O_8$ ($M^+ - 151$): 585.34268. Found: 585.33994. FD-MS *m/z* (relative intensity): 736 (M^+, 100).

6''-Dihydro-3,5-*O*-isopropylidene-14'-*O*-(4-methoxybenzyl)tylonolide (19)——A 4% benzene solution on DDQ (77 μl, 3.1 mg, 0.014 mmol) was added to a stirred ice-cold solution of **18** (10.0 mg, 0.0136 mmol) in CH_2Cl_2 (1.0 ml) and H_2O (0.05 ml), and stirring was continued for 3 h at 0—5 °C. The reaction mixture was poured into aqueous $NaHCO_3$, then the organic layer was washed with aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was purified by silica gel TLC with EtOAc–hexane (1 : 1) to give **19** as a colorless oil (6.0 mg, 75%). ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, d, J=7.0 Hz), 0.89 (3H, t, J=7.0 Hz), 1.10—2.00 (8H, m), 1.18 (3H, d, J=6.5 Hz), 1.40 (3H, s), 1.45 (3H, s), 1.82 (3H, s), 2.34 (2H, t, J=6.5 Hz), 2.80—3.03 (3H, m), 3.40—3.80 (2H, m), 3.46 (2H, d, J=5.0 Hz), 3.81 (3H, s), 3.93 (1H, br s), 4.05 (1H, t, J=3 Hz), 4.20 (1H, m), 4.41 (2H, ABq, J=10.0 Hz), 5.01 (1H, dt, J=4.0, 7.5 Hz), 5.83 (1H, d, J=10.5 Hz), 6.21 (1H, d, J=16.0 Hz), 6.87 (2H, d, J=9.0 Hz), 7.15 (1H, d, J=16.0 Hz), 7.23 (2H, d, J=9.0 Hz). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 1720, 1680. MS *m/z* (relative intensity): 586 (M^+, 0.7), 528 (0.8), 450 (4.3), 392 (2.3), 135 (16), 121 (100). Exact MS *m/z* Calcd for $C_{34}H_{50}O_8$ (M^+): 586.35051. Found: 586.34885.

14'-*O*-(4-Methoxybenzyl)tylonolide Hemiacetal (20)——A CH_2Cl_2 solution (2 ml) of **19** (8.3 mg, 0.014 mmol) and PDC (30 mg, 0.08 mmol) was stirred at room temperature for 6 h. The reaction mixture was directly chromatographed on a silica gel column with ether to give 14'-*O*-(4-methoxybenzyl)-3,5-*O*-isopropylidenetylonolide as a colorless oil (7.5 mg, 91%). ^{1}H-NMR ($CDCl_3$) δ: 0.84 (3H, d, J=6.5 Hz), 1.36 (3H, s), 1.43 (3H, s), 1.81 (3H, d, J=1.0 Hz), 2.70—3.10 (4H, m), 3.46 (2H, d, J=5.0 Hz), 3.81 (3H, s), 3.85—4.15 (2H, m), 4.41 (2H, s), 5.04 (1H, dt, J=4.0, 7.5 Hz), 5.85 (1H, d, J=10.5 Hz), 6.17 (1H, d, J=16.0 Hz), 6.86 (2H, d, J=9.0 Hz), 7.17 (1H, d, J=16.0 Hz), 7.25 (2H, d, J=9.0 Hz), 9.64 (1H, t, J=2.0 Hz). MS *m/z* (relative intensity): 584 (M^+, 0.4), 448 (2.0), 135 (17), 121 (100). Exact MS *m/z* Calcd for $C_{34}H_{48}O_8$ (M^+): 584.33486. Found: 584.33424.

This oil was dissolved in THF (2 ml) and 0.5 N HCl (0.5 ml) and stirred for 1 h at room temperature. The reaction mixture was neutralized with $NaHCO_3$ and extracted with ether. The extract was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with EtOAc–hexane (1 : 1) to give **20** as a colorless oil (5.6 mg, 90%). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=7.5 Hz), 0.97 (3H, t, J=7.0 Hz), 1.10—2.25 (9H, m), 1.23 (3H, d, J=7.0 Hz), 1.80 (3H, d, J=1.0 Hz), 2.27—2.70 (2H, m), 2.75—3.12 (1H, m), 3.49 (2H, d, J=5.0 Hz), 3.60 (1H, d, J=10.5 Hz), 3.77—3.90 (1H, m), 3.81 (3H, s), 4.12 (1H, dd, J=10.0, 4.0 Hz), 4.44 (2H, s), 4.93 (1H, dt, J=2.0, 10.0 Hz), 5.35—5.60 (1H, m), 5.80 (1H, d, J=10.5 Hz), 6.33 (0.8H, d, J=16.0 Hz), 6.37 (0.2H, d, J=16.0 Hz), 6.86 (2H, d, J=9.0 Hz), 7.22 (2H, d, J=9.0 Hz), 7.23 (1H, d, J=16.0 Hz). MS *m/z* (relative intensity): 526 (M^+, 0.5), 508 (0.5), 405 (2.8), 387 (2.0), 207 (2.9), 135 (11), 121 (100). Exact MS *m/z* Calcd for $C_{31}H_{42}O_7$ (M^+): 526.29930. Found: 526.29386.

Tylonolide Hemiacetal (1)——A solution of **20** (5.6 mg, 0.010 mmol) and DDQ (3.5 mg, 0.015 mmol) in CH_2Cl_2 (1 ml) and H_2O (0.05 ml) was stirred at room temperature for 6 h, then poured into aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was subjected to silica TLC with EtOAc–hexane (2 : 1) to give **1** as a colorless solid (3.0 mg, 70%), mp 103 °C. ^{1}H-NMR ($CDCl_3$) δ (500 MHz): 0.95 (3H, t, J=7 Hz, C-17), 1.02 (3H, d, J=7 Hz, C-8'), 1.05 (1H, ddd, J=4, 12, 16 Hz, C-7), 1.23 (3H, d, J=7 Hz, C-4'), 1.60—1.73 (3H, m, C-7(1H), C-16(2H)), 1.55 (1H, dq, J=7, 10 Hz, C-4), 1.80—1.95 (1H, m, C-6'), 1.80 (3H, s, C-12'), 1.94 (1H, dd, J=2, 17 Hz, C-2), 2.01—2.07 (1H, m, C-6), 2.19 (0.23H, dd, J=6, 13 Hz, C-6'), 2.27 (0.77H, dd, J=6, 13 Hz, C-6'), 2.45—2.65 (1H, m, C-8), 2.57 (1H, dd, J=11, 17 Hz, C-2), 2.88 (1H, ddt, J=5, 6, 10 Hz, C-14), 3.67 (1H, dd, J=2, 11 Hz, C-3), 3.68—3.77 (2H, m, C-14'), 3.80 (0.23H, dd, J=4, 10 Hz, C-5), 4.12 (0.77H, dd, J=4, 10 Hz, C-5), 4.93 (1H, dt, J=2.10 Hz, C-15), 5.42 (0.23H, d, J=6 Hz, C-6''), 5.50 (0.77H, dd, J=4, 6 Hz, C-6''), 5.80 (1H, d, J=10 Hz, C-13), 6.33 (0.77H, d, J=15 Hz, C-10), 6.37 (0.23H, d, J=15 Hz, C-10), 7.22 (1H, d, J=15 Hz, C-11). MS *m/z* (relative intensity): 424 (M^+, 2), 406 (17), 393 (12), 388 (12), 191 (39), 121 (100). Exact MS *m/z* Calcd for $C_{23}H_{36}O_7$ (M^+): 424.24605. Found: 424.24538.

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18) The *E,Z*-configuration was determined from the chemical shifts in the NMR spectra (*E*, 6.83; *Z*, 6.73).
19) The di-DMPM product was also obtained in 14% yield, but its secondary DMPM group, not the primary one, was selectively deprotected by the treatment with DDQ at $-20\,^{\circ}C$[6] to give **13** in 70% yield. Therefore, the combined yield of **13** was *ca.* 80%.
20) Other reducing agents gave only unsatisfactory results. The reduction with $LiAlH_4$ or $(iso\text{-}Bu)_2AlH$ (DIBAH) was incomplete due to formation of insoluble precipitates. Because of their fairly strong basicity, $NaBH_4$ and $NaAlH_2(OC_2H_4OCH_3)_2$ (Red-Al) brought about epimerization of the C-8 methyl group.
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Highly Stereoselective Total Synthesis of Pikronolide, the Aglycon of the First Macrolide Antibiotic Pikromycin. Crucial Role of Benzyl-Type Protecting Groups Removable by 2,4-Dichloro-5,6-dicyanobenzoquinone Oxidation[1,2]

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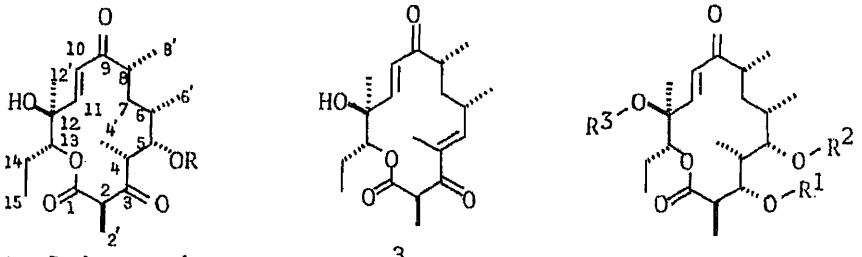
The first total synthesis of pikronolide, the aglycon of pikromycin, isolated as the first macrolide antibiotic, is described. Two segments i (5: C-1—C-10) and ii (6: C-11—C-15) were synthesized highly stereoselectively from D-glucose and coupled by Yamaguchi's method to give the ester (17), which was subjected to macrocyclization by means of the intramolecular Wittig–Horner reaction developed by Nicolaou, and the 14-membered cyclic enone (18) was isolated in excellent yield. Removal of protecting groups and Swern oxidation gave pikronolide (2). In this synthesis, 3,4-dimethoxybenzyl, 4-methoxybenzyl, and benzyl protecting group for hydroxy function played a crucial role.

Keywords——macrolide antibiotic; pikromycin; aglycon; pikronolide; acyclic stereocontrol; protecting group; esterification; Wittig–Horner reaction; 2,4-dichloro-5,6-dicyanobenzoquinone oxidation; stereoselective synthesis

In the preceding papers,[1,3] we reported highly stereoselective syntheses of methynolide and tylonolide to exemplify some of the advantageous features of our synthetic methodology. In the present paper, we report the first total synthesis of pikronolide (**2**), the aglycon of the 14-membered macrolide antibiotic pikromycin (**1**), which was isolated from a strain of *Streptomyces* by Brockmann and Henkel as the first macrolide antibiotic in 1950.[4] However, all attempts at the total synthesis of pikronolide (**2**)[5] as well as pikromycin (**1**) itself during the past 35 years or more have been unsuccessful, because the construction of the β-hydroxyketone system at C-3—C-5[6] of **1** is extremely difficult.[7] Even under very mild hydrolytic conditions (pH 6.5, 60 °C), **1** readily gives the 4,5-anhydro compound, kromycin (**3**).[7b,8] This facile elimination into the α,β-unsaturated ketone system was explained in terms of the *anti*-periplaner disposition of the C-4 hydrogen and the glycoside linkage.[7b] For the total synthesis of **2**, it is essential to avoid such a side reaction. Therefore, the C-3 ketone must be constructed in the final synthetic stage, and we decided to synthesize **4** as a final intermediate. Selection of protecting groups, R^1—R^3, of **4** obviously holds the key to success in the total synthesis of **2**. Differentiation among the three protecting groups and selective deprotection without any effect on the other functional groups and substituents are critical requirements. We chose 3,4-dimethoxybenzyl (DMPM),[9,10] 4-methoxybenzyl (MPM),[10,11] and benzyl (Bn)[10,12] as R^1, R^2, and R^3, respectively. The utility of these protecting groups, removable by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), was shown by their use at crucial synthetic steps described in the preceding papers.[1,3]

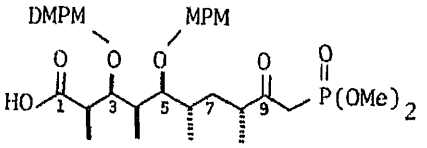
In the methynolide synthesis, the oxidative removal of Bn protection of a tertiary hydroxy function[12a] was successfully applied at the final step.[3c] In the tylonolide synthesis,



1 : R=desosamine
2 : R=H

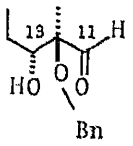
3

4



segment i

5



segment ii

6

Chart 1

not only the selective oxidation of MPM and DMPM protecting groups, but also the selective removal of a Bn protecting group by hydrogenolysis with Raney nickel (Ni)[10,12b] was demonstrated.[1,3d]

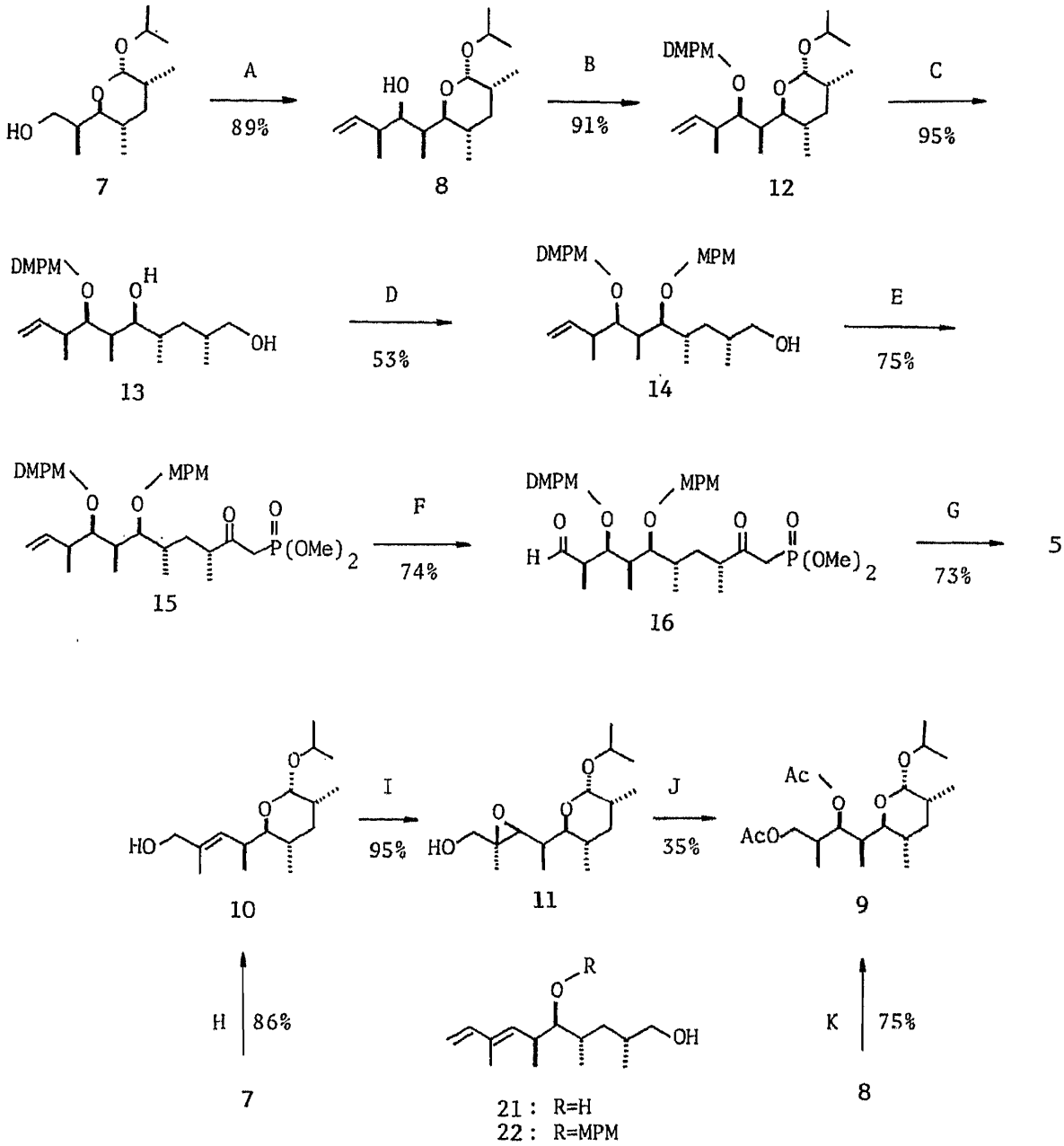
In the present first total synthesis of **2**, the selective removal of DMPM, MPM, and Bn protecting groups again played a decisive role. Segments i (**5**) and ii (**6**) were considered to be the most promising intermediates in the light of our synthetic methodology established in the syntheses of methynolide[3a-c] and tylonolide.[1,3d] In the syntheses of **5** and **6**, DMPM, MPM, and Bn protecting groups for the C-3, C-5 and C-12 hydroxy groups, respectively, were chosen. Segment ii (**6**) was readily synthesized from D-glucose as described in the previous paper,[3c] but the synthesis of the more complex segment i (**5**) was somewhat tedious.

Results and Discussion

The Prelog–Djerassi lactone equivalent compound (**7**), derived from D-glucose as a chiral intermediate for the synthesis of methynolide,[3a] already has four chiral centers corresponding to C-4, C-5, C-6, and C-8, and two additional chiral centers corresponding to C-2 and C-3 were introduced by an *erythro*-selective Cram addition of crotyl-tri-*n*-butyltin.[13] Swern oxidation[14] of the primary alcohol (**7**) readily gave the aldehyde, which was treated with excess boron trifluoride etherate ($BF_3 \cdot Et_2O$; 2.2. eq) and the tin reagent (2.4 eq)[13] at $-90\,^{\circ}C$. The addition of the reagent proceeded quite smoothly to give the expected product (**8**) having all-*syn* configurations of C-2, C-3, and C-4 with excellent yield and stereoselectivity ($> 30 : 1$). The configuration of **8** was confirmed after conversion into the diacetate (**9**), which was also derived from **7** *via* another route involving the Sharpless asymmetric epoxidation.[15]

Oxidative cleavage of the double bond of **8** with ozone and reduction of the resulting aldehyde with sodium borohydride readily gave the diol, which was acetylated to give the diacetate (**9**). An authentic sample of **9** was synthesized as follows. The aldehyde, the Swern oxidation product of **7**, was subjected to the Wittig reaction with a stable ylide, followed by lithium aluminium hydride reduction to give the allyl alcohol (**10**), which was treated with *tert*-butyl hydroperoxide, L-(+)-diethyltartrate, and titanium (IV) isopropoxide,[15] and the expected epoxy alcohol (**11**) was isolated in excellent yield. Reductive ring opening of the epoxide (**11**) took place on treatment with sodium cyanoborohydride in the presence of boron

trifluoride etherate[16] to give mainly the expected 1,3-diol together with the 1,2-diol.[17] Acetylation of the 1,3-diol gave the diacetate (**9**). Both samples of **9** were identical in terms of their nuclear magnetic resonance (NMR) spectra.



(A) 1) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −70 °C→room temperature; 2) MeCH=$CHCH_2SnBu_3$, $BF_3 \cdot Et_2O$, −90 °C→room temperature (B) KH, DMSO, DMPMCl (C) 1) 1 N HCl, THF, 50 °C; 2) $CaCl_2$, $NaBH_4$, EtOH (D) 1) $Me_2C(OMe)_2$, CSA; 2) MPMCl, KCH_2SOMe, DMSO; 3) 0.1 N HCl (E) 1) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −70 °C→room temperature; 2) $MePO(OMe)_2$, *n*-BuLi, THF, −90→−20 °C; 3) PDC, DMF (F) 1) OsO_4, NMO, MeCOMe; 2) $NaIO_4$, MeOH–H_2O (G) CrO_3, H_2SO_4, MeCOMe, −20 °C (H) 1) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −70 °C→room temperature; 2) $Ph_3P=CMeCO_2Et$, $(CH_2Cl)_2$, reflux; 3) $LiAlH_4$, Et_2O, 0 °C (I) (+)DET, TBHP, $(PrO)_4Ti$, CH_2Cl_2–toluene, −23 °C (J) 1) $NaBH_3CN$, $BF_3 \cdot Et_2O$, THF; 2) Ac_2O, Et_3N, DMAP (K) 1) O_3, CH_2Cl_2, −78 °C; 2) $NaBH_4$, MeOH; 3) Ac_2O, Et_3N, DMAP

Chart 2

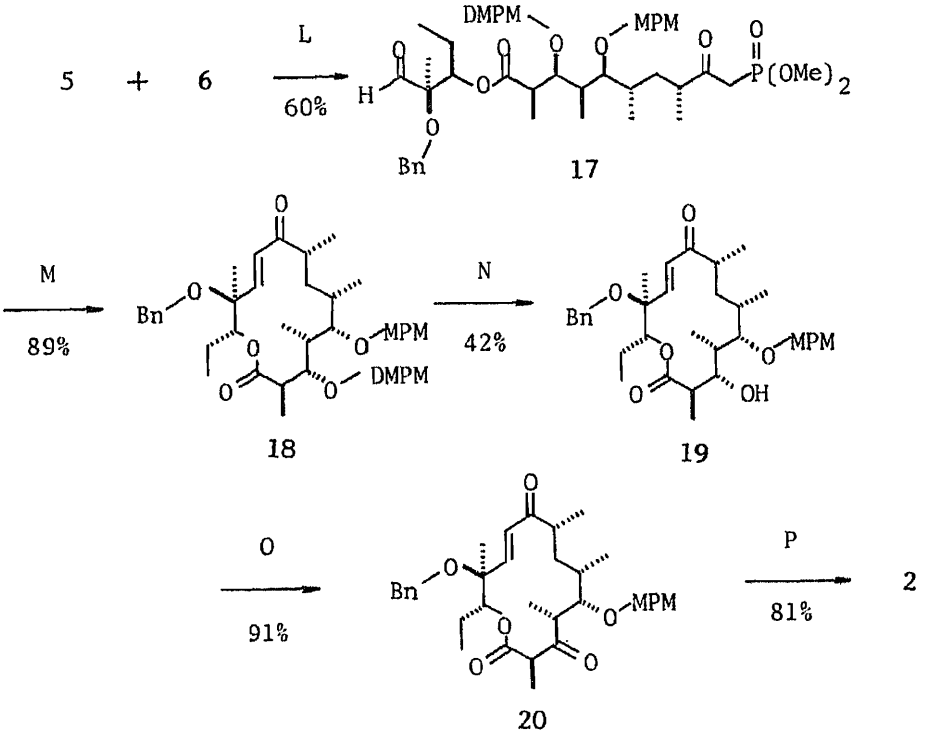
The DMPM protection of the secondary alcohol of **8** was rather difficult. No reaction occurred under usual conditions with sodium hydride and DMPM chloride.[10] Treatment of **8** with a large excess (10—20 eq) of potassium hydride and then DMPM chloride gave the expected DMPM ether (**12**), but the reproducibility of the reaction was poor. However, DMPM protection proceeded very rapidly upon reverse addition of the reagents (see below) to give **12** in excellent yield. The isopropyl protection of **12** was removed with 1 N hydrochloric acid, followed by reduction of the resulting hemiacetal with calcium borohydride to give the open-chain diol (**13**).

The primary alcohol of **13** was first protected as an acetal with the methoxyisopropyl group by treatment with 2,2-dimethoxypropane in the presence of camphorsulfonic acid (CSA), and then protection of the remaining secondary alcohol was examined. The MPM protection of the secondary alcohol at a sterically crowded position was quite difficult, and almost no reaction occurred under usual conditions.[10] Treatment with a large excess of sodium hydride (or dimsyl sodium) and MPM chloride gave only a mixture of dienes, **21** and **22**. However, when the chloride was first added to a dimethyl sulfoxide solution of the above acetal of **13** and then dimsyl potassium was added in two portions (reverse addition), the MPM protection proceeded quite rapidly to give the ether, which was treated with 0.1 N hydrochloric acid to remove the acetal protection, and the expected alcohol (**14**) was isolated in 50—60% yield.

Compound **14** was converted to segment i (**5**) in essentially the same way as described in the previous papers for the syntheses of methynolide[3b,c] and tylonolide.[1] Oxidation of the primary alcohol of **14** by Swern's method readily gave the aldehyde, which was treated with the lithio derivative of dimethyl methylphosphonate[19] at −80 °C followed by oxidation with pyridinium dichromate (PDC) in dimethylformamide (DMF)[20] to give the ketophosphonate (**15**). In order to convert **15** into **5** (segment i), the double bond of **15** was first oxidized directly to the carboxylic acid under Lemieux-von Rudloff's conditions,[21] which were successfully applied in the synthesis of tylonolide,[1] but the yield of **5** was unfortunately less than 10%, because the benzylic positions of the MPM and DMPM protecting groups were not stable enough to this oxidation. Therefore, stepwise oxidation *via* the aldehyde (**16**) was next examined. Oxidation of **15** with osmium tetroxide (OsO_4) in the presence of *N*-methylmorpholine *N*-oxide (NMO) followed by oxidative cleavage of the resulting diol with sodium metaperiodate ($NaIO_4$) gave the aldehyde (**16**), which was oxidized with the Jones reagent at −20 °C for 5 min to give segment i (**5**) in good yield.[22]

Coupling of the two segments i (**5**) and ii (**6**) proceeded smoothly by the Yamaguchi method[23] using 2,4,6-trichlorobenzoyl chloride and 4-dimethylaminopyridine (DMAP) in toluene, and the expected ester (**17**) bearing both aldehyde and ketophosphonate functions was subjected to an intramolecular Wittig–Horner type macrocyclization by Nicolaou's method[24] using a large excess of powdered potassium carbonate and 18-crown-6 in toluene at 80 °C. The cyclization proceeded extremely smoothly and was completed within only 1 h to afford the expected 14-membered enone (**18**) in excellent yield.

For the purpose of conversion of **18** into pikronolide (**2**), it was necessary to remove selectively the DMPM protecting group at C-3 with minimum loss of the MPM and Bn protecting groups and other functional groups. Deprotection of DMPM groups with DDQ usually proceeds with excellent selectivity,[1,9,25] but unfortunately, **18** gave unsatisfactory results with less than 4 : 1 selectivity, and the expected product (**19**) was isolated in poor yield. However, the isolated **19** was converted to **2** very smoothly. Swern oxidation of **19** readily gave the C-3 keto compound (**20**) in excellent yield. Finally, when **20** was further treated with a large excess of DDQ in dichloromethane containing a small amount of water at room temperature, the MPM protecting group at C-5 was removed quite rapidly within 5 min; the clean deprotection of the Bn group at the C-12 proceeded rather slowly and required 19 h to



(L) 2,4,6-$Cl_3C_6H_2COCl$, Et_3N, DMAP, toluene (M) K_2CO_3, 18-crown-6, toluene, 80 °C (N) DDQ, toluene–H_2O, 0 °C (O) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −65→−55 °C (P) DDQ, CH_2Cl_2–H_2O

Chart 3

complete. Surprisingly, during the reaction with DDQ, no trace of kromycin (**3**) was detected, and pikronolide (**2**) was isolated in high yield. The first total synthesis of **2** was thus achieved with a very high overall stereoselectivity (>86%) for the construction of the new chiral centers at C-2, C-4, C-6, C-8, and C-12.[26]

Experimental

Physical data were measured as described in the previous paper.[3a]

(3*S*,4*R*,5*S*)-4-Hydroxy-5-[2(*S*)-isopropyloxy-3(*R*),5(*S*)-dimethyl-6(*S*)-tetrahydroxypropanyl]-3-methylhexane (8)——Dry dimethyl sulfoxide (DMSO) (Me_2SO, 1.23 ml, 1.73 mmol) in CH_2Cl_2 (7 ml) was added dropwise during 15 min to an efficiently stirred solution of oxalyl chloride (0.75 ml, 8.7 mmol) in dry CH_2Cl_2 (15 ml) cooled to below −65 °C under an argon atmosphere. After 15 min at −70 °C, a solution of **4** (1.0 g, 4.34 mmol) was added to the mixture during 10 min. Stirring was continued at −70 °C for 30 min, then Et_3N (4.8 ml, 34 mmol) was added dropwise, and after removal of the cooling bath, the reaction mixture was allowed to warm to room temperature (over *ca.* 1 h). Then H_2O (20 ml) was added. The organic layer was separated, and the aqueous layer was extracted with ether (30 ml×2), and the combined extracts were washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (9 : 1) as the eluant to give the aldehyde (0.95 g, 95%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.83 (3H, d, J=7 Hz), 0.85 (3H, d, J=7 Hz), 1.06 (3H, d, J=7 Hz), 1.09 (3H, d, J=7 Hz), 1.18 (3H, d, J=7 Hz), 2.53 (1H, dq, J=2.5, 7 Hz), 3.74 (1H, sept, J=7 Hz), 4.05 (1H, dd, J=10, 2.5 Hz), 4.59 (1H, d, J=3.5 Hz), 9.68 (1H, s).

A stirred solution of the above aldehyde (0.95 g, 4.16 mmol) in dry CH_2Cl_2 (60 ml), cooled at −93 °C under nitrogen, was treated with $BF_3 \cdot Et_2O$ (1.15 ml, 9.3 mmol) in CH_2Cl_2 (5 ml). After 10 min, crotyl-tri-*n*-butyltin (4 ml, 10 mmol) in CH_2Cl_2 (20 ml) was added to the mixture. The rate of addition of $BF_3 \cdot Et_2O$ and crotyl-tri-*n*-butyltin was controlled to keep the temperature below −90 °C. The mixture was stirred at below −90 °C for 15 min, and the reaction was quenched with saturated NH_4Cl (10 ml). The cooling bath was then removed and the reaction mixture was allowed to warm to room temperature, washed with brine, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was chromatographed on a silica gel column with hexane–EtOAc (30 : 1) as the eluant to afford **8** as a colorless oil (1.1 g, 94%). ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, d, J=7 Hz), 0.82 (3H, d, J=7 Hz), 0.87 (3H, d, J=7 Hz), 1.09 (3H, d, J=6 Hz), 1.10 (3H, d, J=7 Hz), 1.23 (3H, d, J=7 Hz), 1.56—1.88 (2H, m), 1.92 (1H, ddq, J=

2, 1.5, 7 Hz), 2.32 (1H, tq, J=9, 7 Hz), 3.41 (1H, dd, J=9, 1.5 Hz), 3.59 (1H, dd, J=10, 2 Hz), 3.83 (1H, sept, J=7 Hz), 4.67 (1H, d, J=3 Hz), 4.97 (1H, dd, J=10, 2 Hz), 5.03 (1H, dd, J=17, 2 Hz), 5.62 (1H, ddd, J=17, 10, 9 Hz). MS m/z (relative intensity): 267 (M^+-17, 0.15), 225 (6), 169 (60), 100 (100). FD-MS m/z (relative intensity): 285 (M^++1, 28), 229 (100), 178 (17), 169 (15). Exact MS m/z Calcd for $C_{17}H_{33}O_3$ (M^++1): 285.2430. Found: 285.2437. Calcd for $C_{14}H_{25}O_2$ (M^+-59): 225.1855. Found: 225.1866. IR ν_{max}^{neat} cm^{-1}: 3500. $[\alpha]_D^{21}+112°$ (c=1.0, $CHCl_3$).

4(*S*)-[2(*S*)-Isopropyloxy-3(*R*),5(*S*)-dimethyl-6(*S*)-tetrahydropyranyl]-2-methyl-2(*E*)-pentenol (10)——A solution of the aldehyde (40 mg, 0.175 mmol), derived from **7**, and $Ph_3P=C(Me)CO_2Et$ (254 mg, 0.7 mmol) in $(CH_2Cl)_2$ (2 ml) was refluxed for 48 h. The Wittig reagent (254 mg, 0.7 mmol) was added again and refluxing was continued for an additional 24 h. After removal of the solvent *in vacuo*, the residue was purified on a silica gel column with hexane–EtOAc (50 : 1) as the eluant to give the oily α,β-unsaturated ester (51.4 mg, 94%).

A solution of the ester (33 mg, 0.105 mmol) in ether (0.2 ml) was added to a stirred solution of $LiAlH_4$ (6 mg, 0.158 mmol) in ether (1 ml) at 0 °C under an argon atmosphere. After 50 min, H_2O (6 μl), 15% NaOH (6 μl), and H_2O (20 μl) were successively added, and the resulting precipitates were removed by filtration. After evaporation of the solvent, the residue was purified through a short silica gel column with hexane–EtOAc (3 : 1) to afford **10** as a colorless oil (27.3 mg, 91%). ^{1}H-NMR ($CDCl_3$) δ: 0.82 (3H, d, J=7 Hz), 0.84 (3H, d, J=7 Hz), 0.95 (3H, d, J=7 Hz), 1.07 (3H, d, J=7 Hz), 1.12 (3H, d, J=7 Hz), 1.69 (3H, d, J=1.5 Hz), 2.66 (1H, ddq, J=9, 3.5, 7 Hz), 3.37 (1H, dd, J=8.5, 3.5 Hz), 3.80 (1H, sept, J=7 Hz), 3.96 (2H, s), 4.66 (1H, d, J=2 Hz), 5.58 (1H, dq, J=9, 1.5 Hz). IR ν_{max}^{neat} cm^{-1}: 3325. MS m/z (relative intensity): 211 (M^+-75, 6), 171 (67.5), 129 (99), 100 (70), 71 (76), 58 (96), 43 (100). $[\alpha]_D^{1.5}+146.2°$ (c=1.07, $CHCl_3$).

2(*R*),3(*S*)-Epoxy-4(*S*)-[2(*S*)-isopropyloxy-3(*R*),5(*S*)-dimethyl-6(*S*)-tetrahydropyranyl]-2(*S*)-methylpentanol (11)——A solution of L(+)-diethyl tartrate (31 mg, 0.15 mmol) in CH_2Cl_2 (0.4 ml) was added gradually *via* a syringe to a cold solution (−26 °C) of titanium (IV) isopropoxide (30 μl, 0.1 mmol) in CH_2Cl_2 (0.4 ml). After 10 min at −26 °C, **10** (27.3 mg, 0.09 mmol) in CH_2Cl_2 (0.4 ml) and 3 M *tert*-butyl hydroperoxide (63 μl, 0.18 mmol) in toluene were both added dropwise to the solution. The mixture was allowed to stand for 24 h at −23 °C and then saturated Na_2SO_4 (0.2 ml) was added. The reaction mixture was warmed to room temperature, then celite was added with vigorous stirring, and after 1 h, the celite was filtered off. The filtrate was dried over Na_2SO_4 and concentrated *in vacuo* to give an oil, which was chromatographed on a silica gel column with hexane–EtOAc (5 : 1) as the eluant to give **11** as a colorless oil (27 mg, 95%). ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, d, J=7 Hz), 0.82 (3H, d, J=7 Hz), 1.08 (3H, d, J=6 Hz), 1.09 (3H, d, J=7 Hz), 1.17 (3H, d, J=7 Hz), 1.32 (3H, s), 1.22—1.50 (2H, m), 1.50—1.85 (4H, m), 3.16 (1H, d, J=9 Hz), 3.44 (1H, dd, J=10, 2.5 Hz), 3.49 (1H, dd, J=12, 8.5 Hz), 3.69 (1H, dd, J=12, 5 Hz), 3.78 (1H, sept, J=7 Hz), 4.64 (1H, d, J=3.5 Hz). IR ν_{max}^{neat} cm^{-1}: 3400. MS m/z (relative intensity): 284 (M^+-18, 0.3), 244 (5.5), 171 (8.8), 100 (100), 58 (89), 43 (81). $[\alpha]_D^{16.5}+124.7°$ (c=0.97, $CHCl_3$).

(2*S*,3*R*,4*S*)-1,3-Diacetoxy-4-[2(*S*)-isopropyloxy-3(*R*),5(*S*)-dimethyl-6(*S*)-tetrahydropyranyl]-2-methylpentane (9)——(a) Ozone was introduced into a CH_2Cl_2 solution (0.5 ml) of **8** (10 mg, 0.0352 mmol) at −78 °C. After the color of the solution had changed to blue, the reaction was continued for a further 20 min, and then the reaction mixture was treated with a 10% solution of $NaBH_4$ in MeOH (0.1 ml). After 15 min at −78 °C, the mixture was allowed to warm to room temperature, then quenched with saturated NH_4Cl and extracted with CH_2Cl_2. The extract was dried ($MgSO_4$) and concentrated to leave an oil, which was purified on a silica gel column with hexane–EtOAc (3 : 1) to afford the 1,3-diol as a colorless oil (7.6 mg, 75%). ^{1}H-NMR ($CDCl_3$) δ: 0.79 (3H, d, J=6.5 Hz), 0.82 (3H, d, J=6.5 Hz), 0.97 (3H, d, J=6.5 Hz), 1.04 (3H, d, J=6.5 Hz), 1.09 (3H, d, J=6.5 Hz), 1.24 (3H, d, J=6.5 Hz), 1.4—1.5 (2H, m), 1.8—1.92 (2H, m), 2.08 (1H, brs), 3.47 (1H, brs), 3.57 (1H, dd, J=10, 2 Hz), 3.62 (1H, dd, J=5.5, 3 Hz), 3.72 (1H, dd, J=6, 3 Hz), 3.80 (1H, sept, J=6.5 Hz), 4.66 (1H, d, J=3.5 Hz). IR ν_{max}^{neat} cm^{-1}: 3350. MS m/z (relative intensity): 270 (M^+-18, 0.2), 246 (2.5), 229 (3.5), 200 (1.5), 169 (7.1), 129 (15), 100 (100). Exact MS m/z Calcd for $C_{16}H_{30}O_3$ (M^+-18): 270.2194. Found: 270.2178.

The 1,3-diol was acetylated with Ac_2O, Et_3N and DMAP in the usual way to give **9** as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=6 Hz), 0.81 (3H, d, J=7 Hz), 0.84 (3H, d, J=6 Hz), 0.93 (3H, d, J=7 Hz), 1.08 (3H, d, J=6 Hz), 1.15 (3H, d, J=6 Hz), 1.21—1.44 (2H, m), 1.50—1.90 (2H, m), 1.90—2.10 (1H, m), 2.02 (3H, s), 2.06 (3H, s), 2.30 (1H, m), 3.43 (1H, dd, J=10, 2 Hz), 3.81 (1H, dd, J=11.5, 6.5 Hz), 3.83 (1H, sept, J=7 Hz), 3.94 (1H, dd, J=11.5, 8 Hz), 4.65 (1H, d, J=3.5 Hz), 5.25 (1H, dd, J=10.5, 2 Hz). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1752, 1730. $[\alpha]_D^{19}+47.6°$ (c=0.42, $CHCl_3$). MS m/z (relative intensity): 372 (M^+, 0.3), 330 (3.5), 131 (15), 122 (26), 100 (100), 58 (53), 43 (70). Exact MS m/z Calcd for $C_{20}H_{36}O_6$ (M^+): 372.2513. Found: 372.2486.

(b) $NaBH_3CN$ (24 mg, 0.384 mmol) and $BF_3\cdot Et_2O$ (31.5 μl, 0.256 mmol) were added to a stirred solution of **11** (9.6 mg, 0.032 mmol) in tetrahydrofuran (THF) (2.5 ml) under reflux. The reaction mixture was cooled to room temperature, poured into ice-cooled saturated $NaHCO_3$, and extracted with CH_2Cl_2. The extract was washed with brine, and dried over $MgSO_4$. After removal of the solvent *in vacuo*, the residue was chromatographed on a silica gel column with hexane–EtOAc (10 : 1) as the eluant to give two oily fractions. The first fraction gave the 1,3-diol (3.4 mg, 35%), and the second fraction gave the 1,2-diol (2.0 mg, 21%). The 1,3-diol was readily converted to **9** in the usual way.

(3*S*,4*R*,5*S*)-4-(3,4-Dimethoxybenzyloxy)-5-[2(*S*)-isopropyloxy-3(*R*),5(*S*)-dimethyl-6(*S*)-tetrahydroxypyranyl]-3-methylhexene (12)——A 2.5 M potassium dimsyl anion solution [1 ml; prepared from KH (99 mg) and DMSO

(1 ml)] was slowly added dropwise to a DMSO solution (0.7 ml) of DMPM chloride (360 mg, 1.9 mmol) and **8** (68.4 mg, 0.24 mmol) with ice-cooling. After 5 min, the reaction mixture was poured into 0.1 N HCl, and extracted with CH_2Cl_2. The extract was dried over $MgSO_4$, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–EtOAc (30 : 1) as the eluant to give **12** as a colorless oil (94.2 mg, 91%). ^{1}H-NMR ($CDCl_3$) δ: 0.78 (3H, d, J=7 Hz), 0.82 (3H, d, J=7 Hz), 1.02 (3H, d, J=7 Hz), 1.05 (3H, d, J=7 Hz), 1.09 (3H, d, J=6 Hz), 1.18 (3H, d, J=6 Hz), 1.28 (1H, t, J=12 Hz), 1.40 (1H, dt, J=12, 4 Hz), 1.55—1.85 (2H, m), 1.96 (1H, dq, J=2.5, 7 Hz), 2.57 (1H, tq, J=1, 7 Hz), 3.38 (1H, d, J=2.5 Hz), 3.47 (1H, d, J=2.5 Hz), 3.85 (1H, sept, J=7 Hz), 3.86 (3H, s), 3.88 (3H, s), 4.47 (1H, d, J=11 Hz), 4.54 (1H, d, J=11 Hz), 4.67 (1H, d, J=4 Hz), 5.09 (1H, ddd, J=11, 2, 1 Hz), 5.13 (1H, ddd, J=17, 2, 1 Hz), 6.03 (1H, ddd, J=17, 11, 7 Hz), 6.80 (1H, d, J=8 Hz), 6.88 (1H, dd, J=8, 2 Hz), 6.92 (1H, d, J=2 Hz). $[\alpha]_D^{21}$ +56.5° (c=0.92, $CHCl_3$). MS m/z (relative intensity): 434 (M^+, 0.6), 374 (0.8), 166 (12), 151 (100). Exact MS m/z Calcd for $C_{26}H_{42}O_5$ (M^+): 434.3036. Found: 434.3053.

(2*R*,4*S*,5*S*,6*R*,7*R*,8*S*)-7-(3,4-Dimethoxybenzyloxy)-2,4,6,8-tetramethyl-9-decene-1,5-diol (13)——A solution of **12** (0.66 g, 1.52 mmol) in 1 N HCl (4 ml) and THF (12 ml) was stirred at 50 °C for 12 h. After neutralization with solid $NaHCO_3$, the reaction mixture was evaporated to dryness. CH_2Cl_2 and water were added to the residue, and the CH_2Cl_2 layer was separated. The aqueous layer was extracted with CH_2Cl_2 several times, and the organic layers were combined and dried over $MgSO_4$. After evaporation of the solvent, purification of the residue on a silica gel column with hexane–EtOAc (7 : 1) as the eluant afforded recovered **12** (66 mg, 10%) and the lactol as a colorless oil (522 mg, 87%). MS m/z (relative intensity): 392 (M^+, 0.7), 374 (M^+-18, 1), 166 (5), 151 (100). Exact MS m/z Calcd for $C_{23}H_{36}O_5$ (M^+): 392.2564. Found: 392.2566.

A solution of $CaCl_2$ (1 g, 8.8 mmol) in EtOH was cooled at −40 °C, and $NaBH_4$ (0.5 g, 13.3 mmol) in EtOH (20 ml) was added dropwise. NaCl separated out at once as a fine solid. After 30 min, an EtOH solution of the above lactol (520 mg) was added to the resulting $Ca(BH_4)_2$ solution. The reaction mixture was stirred for 3 h at room temperature, then excess $Ca(BH_4)_2$ was decomposed by addition of 1 N HCl, and the mixture was neutralized with Na_2CO_3. After removal of the precipitates by filtration, the filtrate was concentrated *in vacuo*. The residue was extracted with CH_2Cl_2, dried over $MgSO_4$, and evaporated *in vacuo* to leave **13** as a colorless oil (0.51 g, 98%). ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, d, J=7 Hz), 0.90 (3H, d, J=7 Hz), 0.94 (3H, d, J=7 Hz), 1.14 (3H, d, J=7 Hz), 1.5—2.1 (6H, m), 2.64 (1H, dq, J=8, 7 Hz), 3.35 (1H, dd, J=11, 3 Hz), 3.39 (1H, d, J=4 Hz), 3.46 (1H, d, J=3 Hz), 3.59 (1H, dd, J=11, 5.5 Hz), 3.86 (3H, s), 3.87 (3H, s), 4.45 (1H, d, J=11 Hz), 4.66 (1H, d, J=11 Hz), 5.02 (1H, ddd, J=10.5, 1, 0.5 Hz), 5.06 (1H, ddd, J=17.5, 2, 1 Hz), 6.87 (1H, ddd, J=17.5, 10.5, 8 Hz), 6.72—6.80 (3H, m). IR ν_{max}^{neat} cm^{-1}: 3400. $[\alpha]_D^{19.5}$ −46.4° (c=1.38, $CHCl_3$). MS m/z (relative intensity): 394 (M^+, 0.5), 167 (4), 151 (100). Exact MS m/z Calcd for $C_{23}H_{38}O_5$ (M^+): 394.2720. Found: 394.2731.

(2*R*,4*S*,5*R*,6*R*,7*R*,8*S*)-7-(3,4-Dimethoxybenzyloxy)-5-(4-methoxybenzyloxy)-2,4,6,8-tetramethyl-9-decenol (14)——A 2,2-dimethoxypropane (20 ml) solution of **13** (1.2 g, 2.33 mmol) and CSA (40 mg) was stirred at room temperature for 30 min. Et_3N (1 ml) was added to quench the reaction and evaporation of the solvent gave an acetal (1.4 g). This acetal was dissolved in DMSO (14 ml), and MPM chloride (4.3 ml, 10 eq) was added, then a 2.5 M dimsyl potassium solution (14 ml; prepared from KH and DMSO at room temperature) was added dropwise to the mixture with vigorous stirring. The reaction mixture was poured into 0.1 N HCl, and extracted with CH_2Cl_2. The extract was washed with brine, dried, and concentrated. The residue was purified by silica gel column chromatography with hexane–EtOAc (3 : 1) as the eluant to give **14** as a colorless oil (0.82 g, 53%). ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, d, J=7 Hz), 0.95 (3H, d, J=7 Hz), 1.06 (3H, d, J=7 Hz), 1.10 (3H, d, J=7 Hz), 1.38 (1H, m), 1.49 (1H, ddd, J=12.5, 9.5, 3.5 Hz), 1.56 (1H, m), 1.75 (1H, m), 1.85 (1H, m), 2.02 (1H, ddq, J=5.5, 5, 7 Hz), 2.58 (1H, ddq, J=8, 6.5, 7 Hz), 3.17 (1H, t, J=5.5 Hz), 3.28 (1H, dd, J=6.5, 5 Hz), 3.38 (1H, dd, J=11, 6 Hz), 3.50 (1H, dd, J=11, 4.5 Hz), 3.80 (3H, s), 3.87 (6H, s), 4.44 (1H, d, J=11 Hz), 4.49 (2H, s), 4.57 (1H, d, J=11 Hz), 4.99 (1H, ddd, J=10.5, 2, 1 Hz), 5.05 (1H, ddd, J=17.5, 2, 1 Hz), 5.84 (1H, ddd, J=17.5, 10.5, 8 Hz), 6.81—6.91 (5H, m), 6.86 (2H, d, J=9 Hz), 7.25 (2H, d, J=9 Hz). IR ν_{max}^{neat} cm^{-1}: 3450. $[\alpha]_D^{23.5}$ −11.8° (c=1.32, $CHCl_3$). MS m/z (relative intensity): 393 (M^+-121, 3), 363 (0.8), 227 (7), 167 (16), 151 (100), 121 (66). FI-MS m/z (relative intensity): 515 (M^++1, 33.5), 514 (M^+, 100), 122 (13), 121 (8). Exact MS m/z Calcd for $C_{23}H_{37}O_5$ (M^+-121): 393.2641. Found: 393.2656.

Dimethyl (3*R*,5*S*,6*R*,7*R*,8*R*,9*S*)-8-(3,4-Dimethoxybenzyloxy)-6-(4-methoxybenzyloxy)-2-oxo-3,5,7,9-tetramethyl-10-undecenylphosphonate (15)——Dry DMSO (0.42 ml, 5.9 mmol) in CH_2Cl_2 (1 ml) was added to a stirred solution of oxalyl chloride (0.21 ml, 2.33 mmol) in CH_2Cl_2 (5 ml) at −70 °C during 5 min. After 15 min, **14** (300 mg, 0.58 mmol) in dry CH_2Cl_2 (3 ml) was added. Stirring was continued for 30 min at −70 °C, then Et_3N (1 ml) was added dropwise, and after 15 min, the reaction mixture was allowed to warm to room temperature. After 1 h at room temperature, the reaction mixture was quenched with H_2O, and extracted with ether. The extract was washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (10 : 1) to afford (2*R*,4*S*,5*S*,6*R*,7*R*,8*S*)-7-(3,4-dimethoxybenzyloxy)-5-(4-methoxybenzyloxy)-2,4,6,8,-tetramethyl-9-hexenal as a colorless oil (284 mg, 95%). ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, d, J=7 Hz), 1.04 (3H, d, J=7 Hz), 1.09 (3H, d, J=7 Hz), 1.13 (3H, d, J=7 Hz), 1.56—2.16 (2H, m), 2.40 (1H, m), 2.52 (1H, m), 3.14 (1H, dd, J=7, 4 Hz), 3.24 (1H, dd, J=7, 4 Hz), 3.80 (3H, s), 3.88 (6H, s), 4.38 (1H, d, J=11 Hz), 4.47 (2H, s), 4.60 (1H, d, J=11 Hz), 5.00 (1H, ddd, J=10.5, 2, 1 Hz), 5.04 (1H, ddd, J=18, 2, 1 Hz), 5.80 (1H, ddd, J=18, 10.5, 8 Hz), 6.86 (2H, d, J=9 Hz), 6.88 (3H, d, J=6 Hz), 7.24 (2H, d, J=9 Hz), 9.52 (1H, d, J=3 Hz). IR ν_{max}^{neat} cm^{-1}: 1720. MS m/z (relative

intensity): 391 (M^+-121, 3), 361 (1.3), 167 (14), 151 (100), 121 (53). Exact MS *m/z* Calcd for $C_{23}H_{35}O_5$ (M^+-121): 391.2485. Found: 391.2493.

A 1.6 M *n*-BuLi solution in hexane (2.25 ml) was added to a stirred solution of dimethyl methylphosphonate (0.52 ml, 4.8 mmol) in THF at −90 °C. After 45 min, the above aldehyde (0.16 g, 1.2 mmol) in THF (3 ml) was added dropwise, and the reaction mixture was gradually warmed to −20 °C during 8 h. After the reaction had been quenched with saturated NH_4Cl solution, the whole mixture was extracted with ether, and the extract was washed with brine, dried over $MgSO_4$, and evaporated *in vacuo* to give the β-hydroxyphosphonate as an oil (0.69 g, 91%). MS *m/z* (relative intensity): 515 (M^+-121, 2.4), 485 (M^+-151, 0.5), 349 (4), 235 (16), 151 (100), 121 (69). Exact MS *m/z* Calcd for $C_{26}H_{44}O_8P$ (M^+-121): 515.2775. Found: 515.2791.

PDC (2 g, 5.5 mmol) was added to a stirred solution of the β-hydroxyphosphonate (0.69 g, 1.1 mmol) in DMF (12 ml) at room temperature. After 5.5 h, the reaction mixture was poured into H_2O and then extracted with ether. The extract was washed with brine, dried ($MgSO_4$), and concentrated *in vacuo*, and the residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 2) to give 597 mg (86.5%) of the ketophosphonate (**15**) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, d, J=7 Hz), 1.06 (3H, d, J=7 Hz), 1.12 (3H, d, J=7 Hz), 1.15 (3H, d, J=7 Hz), 1.65—1.80 (1H, m), 1.90 (1H, ddd, J=14, 10.5, 2.5 Hz), 2.04 (1H, ddq, J=7.5, 7, 3.5 Hz), 2.56 (1H, ddq, J=8, 7.5, 7 Hz), 2.90 (1H, ddq, J=10.5, 7, 3.5 Hz), 3.09 (1H, dd, J=23, 14.5 Hz), 3.19 (1H, dd, J=23, 14.5 Hz), 3.19 (1H, dd, J=7, 3.5 Hz), 3.33 (1H, dd, J=7.5, 3.5 Hz), 3.77 (1H, d, J=11 Hz), 3.78 (1H, d, J=11 Hz), 3.80 (3H, s), 3.87 (3H, s), 3.88 (3H, s), 4.40 (1H, d, J=11 Hz), 4.45 (1H, d, J=11 Hz), 4.50 (1H, d, J=11 Hz), 4.58 (1H, d, J=11 Hz), 4.99 (1H, dd, J=10.5, 2 Hz), 5.05 (1H, ddd, J=17.5, 2, 1 Hz), 5.91 (1H, ddd, J=17.5, 10.6, 8 Hz), 6.9—6.96 (5H, m), 7.1—7.3 (2H, m). IR ν_{max}^{neat} cm^{-1}: 1710. $[\alpha]_D^{25}$ +15° (c=1.11, $CHCl_3$). MS *m/z* (relative intensity): 513 (M^+-121, 1.9), 483 (0.4), 347 (6.6), 311 (5.2), 233 (7.7), 151 (100), 121 (60).

(2*R*,3*S*,4*R*,5*S*,6*S*,8*R*)-3-(3,4-Dimethoxybenzyloxy)-10-dimethoxyphosphono-5-(4-methoxybenzyloxy)-2,4,6,8-tetramethyl-9-oxo-decanal (16)——NMO (0.34 g, 2.52 mmol) was added to a stirred solution of **15** (0.53 g, 0.84 mmol) in a 1 : 4 mixture of acetone and H_2O (6 ml) at room temperature and then a *tert*-BuOH solution of OsO_4 (0.02 eq) was further added. After 1 h, 10% $Na_2S_2O_4$ solution (3 ml) and celite was added with vigorous stirring, and the celite was filtered off. The filtrate was concentrated *in vacuo* and the residue was extracted with CH_2Cl_2. The extract was washed with 0.1 N HCl and brine, dried over $MgSO_4$, and evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–MeOH (20 : 1) as the eluant to give 467 mg (74%) of the diol as an oil.

The diol was dissolved in MeOH (6 ml) and treated with $NaIO_4$ (282 mg, 1.32 mmol) in H_2O (3 ml) at 0 °C. The reaction mixture was stirred for 30 min at room temperature, then diluted with water, and extracted with CH_2Cl_2. The extract was dried over $MgSO_4$, and concentrated *in vacuo* to give **16** as an oil (447 mg, 100%). ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, d, J=7 Hz), 1.06 (3H, d, J=7 Hz), 1.13 (3H, d, J=7 Hz), 1.17 (3H, d, J=7 Hz), 2.5—3.24 (4H, m), 2.98 (1H, dd, J=22.5, 14 Hz), 3.21 (1H, dd, J=22.5, 14 Hz), 3.75 (3H, d, J=11 Hz), 3.77 (3H, d, J=11 Hz), 3.80 (3H, s), 3.87 (6H, s), 4.32 (1H, d, J=11 Hz), 4.39 (1H, d, J=11 Hz), 4.46 (1H, d, J=11 Hz), 4.53 (1H, d, J=11 Hz), 6.83 (3H, s), 6.87 (2H, d, J=9 Hz), 7.24 (2H, d, J=9 Hz), 9.76 (1H, d, J=1 Hz). IR ν_{max}^{neat} cm^{-1}: 1715, 1710.

(2*R*,3*S*,4*R*,5*S*,6*S*,8*R*)-3-(3,4-Dimethoxybenzyloxy)-10-dimethoxyphosphono-5-(4-methoxybenzyloxy)-2,4,6,8-tetramethyl-9-oxodecanoic Acid (5)——A stirred acetone solution (5.7 ml) of **16** (242 mg, 0.38 mmol), cooled at −20 °C, was treated dropwise with 2.67 M Jones reagent (285 μl, 0.38 mmol). After 5 min, 2-propanol (1 ml) was added to quench the reaction, and the reaction mixture was allowed to warm to room temperature. Ether and H_2O were then added. The organic layer was separated, and the aqueous layer was extracted with ether and CH_2Cl_2. The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo*. The residue was dissolved again in ether, and then extracted with saturated $NaHCO_3$. The alkaline extract was neutralized with saturated NH_4Cl and extracted with CH_2Cl_2. The extract was dried ($MgSO_4$), and concentrated *in vacuo* to afford **5** (180 mg, 72.5%) as a colorless oil, which was used for the next reaction without further purification. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, d, J=7 Hz), 1.09 (3H, d, J=7 Hz), 1.11 (3H, d, J=7 Hz), 1.34 (3H, d, J=7 Hz), 1.63 (1H, m), 1.82 (1H, m), 1.94 (1H, ddq, J=3, 1, 7 Hz), 2.67 (1H, dq, J=0.5, 7 Hz), 2.76 (1H, dq, J=9.5, 7 Hz), 3.08 (1H, dd, J=8.5, 1 Hz), 3.18 (1H, br s), 3.25 (1H, br s), 3.55 (1H, d, J=9.5 Hz), 3.78 (3H, d, J=11.5 Hz), 3.78 (3H, s), 3.84 (3H, d, J=11.5 Hz), 3.86 (3H, s), 3.87 (3H, s), 4.32 (1H, d, J=11 Hz), 4.46 (1H, d, J=11 Hz), 4.48 (1H, d, J=11 Hz), 4.67 (1H, d, J=11 Hz), 6.83 (2H, d, J=8.5 Hz), 6.82—6.90 (3H, m), 7.20 (2H, d, J=8.5 Hz). IR ν_{max}^{neat} cm^{-1}: 1735, 1710. $[\alpha]_D^{16.5}$ 0° (c=2.63, $CHCl_3$). MS *m/z* (relative intensity): 531 (M^+-121, 1.6), 501 (0.4), 329 (8.6), 233 (8.6), 180 (12), 151 (69), 121 (100). Exact MS *m/z* Calcd for $C_{25}H_{40}O_{10}P$ (M^+-121): 531.2360. Found: 531.2330.

(1*R*,2*R*)-2-Benzyloxy-1-ethyl-2-formylpropyl (2*R*,3*S*,4*R*,5*S*,6*S*,8*R*)-3-(3,4-Dimethoxybenzyloxy)-10-dimethoxyphosphono-5-(4-methoxybenzyloxy)-2,4,6,8-tetramethyl-9-oxodecanate (17)——2,4,6-Trichlorobenzoyl chloride (30.5 μl, 0.21 mmol) was added dropwise to a stirred solution of **5** (113 mg, 0.173 mmol) and Et_3N (29 μl, 0.19 mmol) in THF (3 ml) at room temperature. After 1 h, precipitated $Et_3N \cdot HCl$ was filtered off and the filtrate was evaporated *in vacuo* to leave an oil, which was dissolved in toluene (2 ml). To this stirred solution, a mixture of **6** (77 mg, 0.346 mmol) and DMAP (21 mg, 0.173 mmol) in toluene (0.83 ml) was added. After 4 h, the reaction mixture was diluted with ether, washed with brine and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (1 : 2) to give **17** as a colorless oil (88.5 mg, 60%).

^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7 Hz), 0.91 (3H, d, J=7 Hz), 1.06 (3H, d, J=7 Hz), 1.11 (3H, d, J=7 Hz), 1.29 (3H, d, J=7 Hz), 1.32 (3H, s), 1.61 (2H, m), 1.77 (2H, m), 1.90 (2H, m), 2.75—2.90 (2H, m), 3.09 (1H, dd, J=22.5, 14.5 Hz), 3.15 (1H, dd, J=22.5, 14.5 Hz), 3.15 (1H, dd, J=6, 4 Hz), 3.74 (3H, d, J=11.5 Hz), 3.76 (3H, d, J=11.5 Hz), 3.79 (3H, s), 3.85 (3H, s), 3.86 (3H, s), 4.41 (3H, d, J=11 Hz), 4.49 (1H, d, J=11 Hz), 4.54 (1H, d, J=11 Hz), 4.57 (1H, d, J=11 Hz), 5.22 (1H, dd, J=11, 3.5 Hz), 6.78—6.88 (3H, m), 6.85 (2H, d, J=9 Hz), 7.25 (2H, d, J=9 Hz), 7.30 (5H, m), 9.62 (1H, s). IR ν_{max}^{neat} cm^{-1}: 1740, 1735, 1710. $[\alpha]_D^{12}$ +20.6° (c=1.42, $CHCl_3$). MS m/z (relative intensity): 735 (M^+−121, 1), 705 (0.2), 569 (3.6), 329 (9), 233 (11), 180 (7), 151 (91), 121 (100), 91 (36). FI-MS m/z (relative intensity): 856 (M^+, 36.6), 736 (73), 166 (100).

(3*R*,4*S*,5*R*,6*S*,7*S*,9*R*,13*R*,14*R*)-13-Benzyloxy-4-(3,4-dimethoxybenzyloxy)-14-ethyl-6-(4-methoxybenzyloxy)-3,5,7,9,13-pentamethyl-1-oxacyclotetradec-11(*E*)-ene-2,10-dione (18)——A solution of **17** (2.9 mg) in toluene (1.5 ml) was added dropwise to a stirred suspension of K_2CO_3 (2.8 mg, 0.02 mmol) and 18-crown-6 (10.7 mg, 0.04 mmol) in toluene (2 ml) during 30 min at 80 °C. After 1 h at 80 °C, the reaction mixture was allowed to cool to room temperature, quenched by addition of saturated NH_4Cl (5 ml), and poured into ether (20 ml). This ether solution was washed with saturated KCl, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–EtOAc (3 : 1) as the eluent to give **18** as a colorless oil (2.2 mg, 89%). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=7 Hz), 0.92 (3H, d, J=6 Hz), 1.07 (3H, d, J=7 Hz), 1.16 (3H, d, J=7 Hz), 1.20—1.30 (1H, m), 1.30 (3H, d, J=7 Hz), 1.39 (3H, s), 1.45—1.60 (1H, m), 1.76 (1H, t, J=12 Hz), 2.02 (1H, ddq, J=14, 7.5, 2.5 Hz), 2.08 (1H, m), 2.50—2.65 (1H, m), 2.69 (1H, dq, J=6, 7 Hz), 3.07 (1H, d, J=8 Hz), 3.40 (1H, dd, J=6, 2.5 Hz), 3.79 (3H, s), 3.86 (3H, s), 3.88 (3H, s), 4.30 (2H, d, J=11 Hz), 4.31 (1H, d, J=11 Hz), 4.41 (1H, d, J=11 Hz), 4.46 (1H, d, J=11 Hz), 4.68 (1H, d, J=11 Hz), 5.01 (1H, dd, J=11, 2 Hz), 6.37 (1H, d, J=15.5 Hz), 6.80—6.90 (3H, m), 7.00 (1H, d, J=15.5 Hz), 7.19 (1H, d, J=9 Hz), 7.29 (1H, d, J=9 Hz), 7.29 (5H, m). IR ν_{max}^{neat} cm^{-1}: 3450, 1735, 1700. $[\alpha]_D^{16.5}$ −4.2° (c=1.64, $CHCl_3$). MS m/z (relative intensity): 609 (M^+−121, 3), 579 (1), 335 (4), 167 (7), 151 (100), 121 (86), 91 (42). FI-MS m/z (relative intensity): 730 (M^+, 100), 580 (14), 136 (12). Exact MS m/z Calcd for $C_{36}H_{49}O_8$ (M^+−121): 609.3429. Found: 609.3410.

(3*R*,4*S*,5*R*,6*S*,7*S*,9*R*,13*R*,14*R*)-13-Benzyloxy-14-ethyl-4-hydroxy-6-(4-methoxybenzyloxy)-3,5,7,9,13-pentamethyl-1-oxacyclotetradec-11(*E*)-ene-2,10-dione (19)——DDQ (7.6 mg, 0.032 mmol) was added to a stirred ice-cold solution of **18** (24.4 mg, 0.0334 mmol) in toluene (1 ml) and H_2O (0.05 ml). After being stirred for 4.5 h at 0 °C, the reaction mixture was quenched by addition of saturated $NaHCO_3$, and extracted with EtOAc. The extract was dried ($MgSO_4$) and concentrated to leave an oil, which was subjected to preparative thin layer chromatography (TLC) on silica gel (benzene : EtOAc = 15 : 1, three times development) to give **19** (6.4 mg, 33%, net 42%). ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, t, J=7.5 Hz), 1.02 (6H, d, J=7 Hz), 1.11 (3H, d, J=7 Hz), 1.28 (3H, d, J=7 Hz), 1.38 (3H, s), 1.90 (1H, m), 1.94 (1H, ddd, J=14, 7, 2 Hz), 2.66 (1H, dq, J=8.5, 7 Hz), 2.70 (1H, d, J=2.5 Hz), 2.78 (1H, dquint, J=8, 7 Hz), 3.51 (1H, t, J=4.5 Hz), 3.75 (1H, d, J=8.5 Hz), 3.81 (1H, s), 4.30 (1H, d, J=11 Hz), 4.41 (1H, d, J=11 Hz), 4.42 (1H, d, J=11 Hz), 4.45 (1H, d, J=11 Hz), 5.14 (1H, dd, J=11, 2.5 Hz), 6.28 (1H, d, J=16.5 Hz), 6.79 (1H, d, J=16.5 Hz), 6.89 (2H, d, J=9 Hz), 7.27 (2H, d, J=9 Hz), 7.23—7.37 (5H, m). IR ν_{max}^{neat} cm^{-1}: 3450, 1730, 1695. $[\alpha]_D^{13.5}$ −2.6° (c=1.0, $CHCl_3$). MS m/z (relative intensity): 472 (M^+−108, 0.15), 401 (0.7), 336 (4.8), 121 (100), 91 (34.5). FI-MS m/z (relative intensity): 580 (M^+, 100), 121 (11.7). Exact MS m/z Calcd for $C_{28}H_{40}O_5$ (M^+−108): 472.2825. Found: 472.2833.

(3*R*,5*R*,6*S*,7*S*,9*R*,13*R*,14*R*)-14-Ethyl-13-benzyloxy-6-(4-methoxybenzyloxy)-3,5,7,9,13-pentamethyl-1-oxacyclotetradec-11(*E*)-ene-2,4,10-trione (20)——A solution of DMSO (16.3 μl, 0.23 mmol) in CH_2Cl_2 (50 μl) was added to a stirred solution of oxalyl chloride (10 μl, 0.164 mmol) in dry CH_2Cl_2 (0.2 ml) at −70 °C. After 15 min, a CH_2Cl_2 solution of **19** (10 mg, 0.017 mmol) was added to the reaction mixture. Stirring was continued for 30 min at −65 °C, and then Et_3N (48 μl, 0.345 mmol) was added. After 70 min at −65——−55 °C, the reaction mixture was quenched with saturated NH_4Cl, then allowed to warm to room temperature during 1 h, and diluted with CH_2Cl_2. The CH_2Cl_2 solution was washed with brine, and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified on a silica gel column with hexane–EtOAc (3 : 1) as the eluant to afford **20** as a viscous oil (9.1 mg, 91%). ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.5 Hz), 1.01 (3H, d, J=7 Hz), 1.08 (3H, d, J=6.5 Hz), 1.27 (3H, d, J=7.5 Hz), 1.38 (3H, d, J=7 Hz), 1.40 (3H, s), 1.89 (1H, m), 2.75 (1H, dq, J=14, 7 Hz), 2.77 (1H, dq, J=6.5, 7 Hz), 3.80 (3H, s), 3.83 (1H, q, J=7 Hz), 3.93 (1H, dd, J=13.5, 6.5 Hz), 4.32 (1H, d, J=11.5 Hz), 4.43 (1H, d, J=11.5 Hz), 4.50 (2H, s), 5.09 (1H, dd, J=11, 2.5 Hz), 6.20 (1H, d, J=16.5 Hz), 6.73 (1H, d, J=16.5 Hz), 6.87 (1H, d, J=9 Hz), 7.27 (1H, d, J=9 Hz), 7.32 (5H, m). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1745, 1715, 1695, 1630. MS m/z (relative intensity): 578 (M^+, 0.1), 470 (0.8), 399 (0.8), 334 (4), 163 (5), 121 (100), 91 (42). FI-MS m/z (relative intensity): 578 (M^+, 100), 138 (22), 122 (24). Exact MS m/z Calcd for $C_{35}H_{46}O_7$ (M^+): 578.3244. Found: 578.3255.

Pikronolide (2)——DDQ (22 mg, 0.097 mmol) was added to a stirred solution of **20** (5.4 mg, 0.0934 mmol) in a mixture of CH_2Cl_2 and H_2O (20 : 1, 0.5 ml) at room temperature. After 19 h, the reaction mixture was directly chromatographed on a silica gel column with hexane–EtOAc (2 : 1—1 : 1) as the eluant to give **2** as a colorless solid (2.7 mg, 81%), mp 140—141.5 °C (EtOAc–hexane). ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, J=7 Hz, C-15), 1.02 (3H, d, J=7 Hz, C-6′), 1.11 (3H, d, J=6 Hz, C-8′), 1.19—1.31 (1H, m, C-4′), 1.24 (3H, d, J=7 Hz, C-4′), 1.34 (3H, s, C-12′), 1.40—1.60 (1H, m, C-14), 1.44 (3H, d, J=7 Hz, C-2′), 1.45—1.59 (1H, m, C-7), 1.79 (1H, ddq, J=14, 2.5, 7 Hz, C-14), 1.94 (1H, m, C-6), 1.95 (1H, d, J=4.5 Hz, C-5 OH), 2.83 (1H, dq, J=12, 6 Hz, C-8), 2.93 (1H, dq, J=5.5, 7 Hz,

C-4), 2.94 (1H, s, C-12 OH), 3.79 (1H, q, $J=7$ Hz, C-2), 3.97 (1H, ddd, $J=5.5$, 4.5, 4 Hz, C-5), 5.00 (1H, dd, $J=11$, 2.5 Hz, C-13), 6.30 (1H, d, $J=16$ Hz, C-10), 6.71 (1H, d, $J=16$ Hz, C-11). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 3450, 1740, 1695, 1635. $[\alpha]_D^{18.5}+66.3°$ ($c=0.187$, MeOH). MS *m/z* (relative intensity): 368 (M^+, 0.6), 350 (14), 310 (3.4), 292 (4.6), 267 (4.2), 254 (7.5), 178 (47), 122 (100), 109 (85). Exact MS *m/z* Calcd for $C_{20}H_{32}O_6$ (M^+): 368.2198. Found: 368.2196

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26) The stereoselectivities at C-4, C-6, C-8, and C-12 were reported in the previous paper.[3c]

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Smiles Rearrangement in Isoquinolinium Salts

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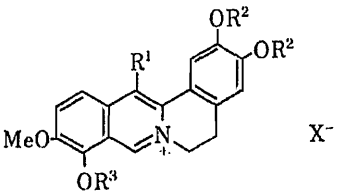
Hydrazinolysis of 5,6-dihydro-2,3,10-trimethoxy-13-methyl-9-(2-phthalimidoethoxy)dibenzo-[*a*,*g*]quinolizinium bromide (**2**) and 7-methoxy-1-(3,4-dimethoxybenzyl)-2-methyl-6-(2-phthalimidoethoxy)isoquinolinium bromide (**17**) gave 5,6-dihydro-9-(2-hydroxyethyl)amino-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium bromide (**3**) and 6-(2-hydroxyethyl)amino-7-methoxy-1-(3,4-dimethoxybenzyl)-2-methylisoquinolinium bromide (**18**), respectively, *via* the Smiles rearrangement. An additional example of these rearrangements is also described.

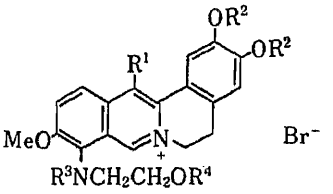
Keywords——Smiles rearrangement; protoberberinium salt; isoquinolinium salt; tetrahydroprotoberberine

There are few papers dealing with Smiles rearrangement of heterocyclic compounds.[1] During the course of our synthetic studies on a series of dehydrocorydaline (**1**) derivatives (protoberberinium salts) having anti-peptic ulcer activity,[2] it was found that hydrazinolysis of the phthalimido derivative (**2**) unexpectedly gave 5,6-dihydro-9-(2-hydroxyethyl)amino-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium bromide (**3**) instead of the expected 9-(2-aminoethoxy)-5,6-dihydro-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium bromide (**4**) (Chart 1).[3] The formation of **3** from **2** was interpreted in terms of the Smiles rearrangement[4] *via* an intermediate (a) as shown in Chart 2. The C_9-position of the protoberberinium salt is activated by the quaternary nitrogen atom (N_7). It may be presumed that the partial structure required for this rearrangement in a protoberberinium salt exists in the isoquinolinium moiety as shown in Chart 2. Thus, we examined this rearrangement in other compounds having the isoquinolinium moiety as a partial structure. This paper presents the details of the first example of the Smiles rearrangement in an isoquinolinium salt (**17**) and protoberberinium salts (**2** and **11**).

In an attempt to synthesize the 2-aminoethoxy derivative (**4**) as an analog of **1**, 9-demethyldehydrocorydaline (**5**)[2] was treated with sodium hydroxide to give a phenol betaine (**6**), which was led to a phthalimido derivative (**2**) by refluxing with 2-bromoethylphthalimide in dioxane. The treatment of **2** with hydrazine hydrate in refluxing ethanol, followed by treatment with hydrobromic acid, gave the unexpected (2-hydroxyethyl)amino derivative (**3**) *via* the Smiles rearrangement almost quantitatively. The structure of **3** was deduced from the following evidence. On sodium borohydride ($NaBH_4$) reduction, **3** gave a tetrahydro derivative (**7**) whose mass spectrum (MS) exhibited intense ion peaks at *m/z* 398 (M^+), 353, 207 and 192 as shown in Chart 1. The presence of the ion peak at *m/z* 353 ($M^+ - CH_2CH_2OH$) can not be explained by mass fragmentation of a tetrahydro derivative of the expected compound (**4**). Acetylation of **3** with acetic anhydride–pyridine at room temperature afforded the O-acetate (**8**), whose infrared (IR) spectrum exhibited carbonyl bands at 1740 cm^{-1}. On refluxing with acetic anhydride, **3** gave the O, N-diacetate (**9**), and its IR spectrum showed carbonyl bands at 1665 and 1735 cm^{-1}. During the course of repeated recrystallization from ethanol, **9** was partially hydrolyzed to give the N-acetate (**10**) having carbonyl bands at 1660 cm^{-1}. As shown in Table I, the ultraviolet (UV) and visible absorption spectra of **3** and **8**



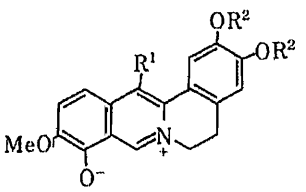
X⁻



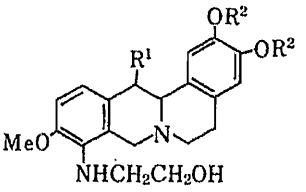
Br⁻

1 : $R^1=R^2=R^3=Me$, X=Cl
2 : $R^1=R^2=Me$, $R^3=CH_2CH_2N$(phthalimido), X=Br
4 : $R^1=R^2=Me$, $R^3=CH_2CH_2NH_2$, X=Br
5 : $R^1=R^2=Me$, $R^3=H$, X=Cl
11 : $R^1=H$, R^2, $R^2=-CH_2-$, $R^3=CH_2CH_2N$(phthalimido), X=Br
12 : $R^1=H$, R^2, $R^2=-CH_2-$, $R^3=H$, X=Cl

3 : $R^1=R^2=Me$, $R^3=R^4=H$
8 : $R^1=R^2=Me$, $R^3=H$, $R^4=Ac$
9 : $R^1=R^2=Me$, $R^3=R^4=Ac$
10 : $R^1=R^2=Me$, $R^3=Ac$, $R^4=H$
14 : $R^1=R^3=R^4=H$, R^2, $R^2=-CH_2-$



6 : $R^1=R^2=Me$
13 : $R^1=H$, R^2, $R^2=-CH_2-$



7 : $R^1=R^2=Me$
15 : $R^1=H$, R^2, $R^2=-CH_2-$

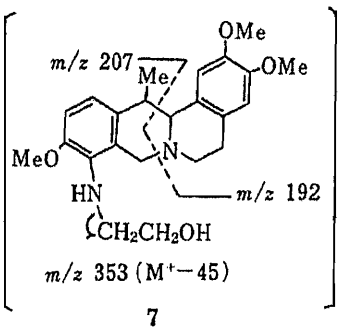


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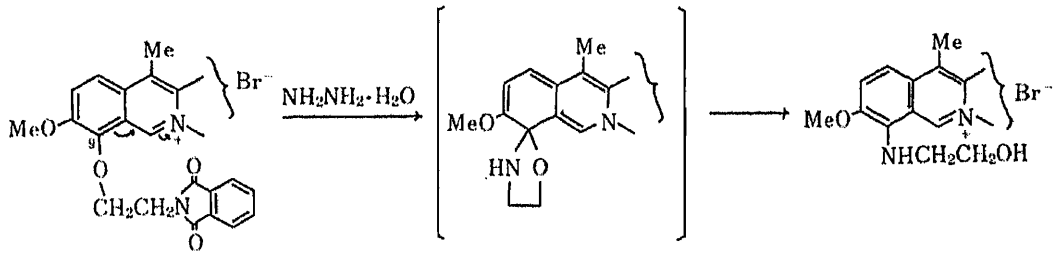
Chart 1



2 (a) 3

Chart 2

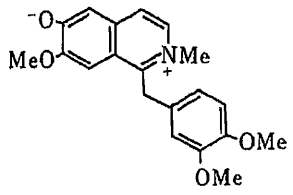
showed bathochromic shifts in comparison with that of **1**, indicating the presence of an NH-chromophore at the C_9-position in accordance with the data described above.

In a similar hydrazinolysis, the berberine derivative, 5,6-dihydro-10-methoxy-2,3-methylenedioxy-9-(2-phthalimidoethoxy)dibenzo[*a*, *g*]quinolizinium bromide (**11**), which was obtained from 9-demethylberberinium chloride (**12**) *via* berberine phenol betaine (**13**), underwent the Smiles rearrangement on treatment with hydrazine hydrate in refluxing methanol to give a (2-hydroxyethyl)amino derivative (**14**). The structure of **14** was fully supported by the spectral data of its tetrahydro derivative (**15**) in comparison with those of **7**.

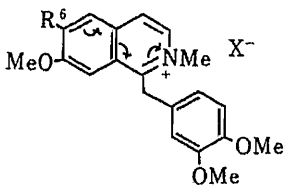
On the other hand, the C_6-position of an isoquinolinium salt is activated by the presence of the quaternary nitrogen atom (N_2). Therefore, it was assumed that the Smiles rearrangement might occur at the C_6-position of an isoquinolinium salt, by analogy with that at the C_9-position of the protoberberinium salts (Chart 3). To test this assumption, the papaverine

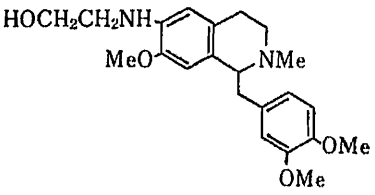
TABLE I. Ultraviolet and Visible Absorption Data

Compd. No.	λ_{max} (log ε) (in EtOH)
1	231 (4.46), 267 (4.52), 340 (4.43), 425 (3.81)
3	232 (4.50), 283 (4.47), 346 (4.17), 480 (3.86)
8	232 (4.52), 283 (4.49), 346 (4.22), 479 (3.85)
18	360 (4.40)
20	310 (4.05)

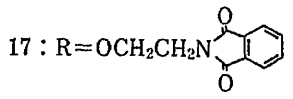


16





21



17 : R=OCH_2CH_2N(phthalimido), X=Br

18 : R=$NHCH_2CH_2OH$, X=Br

19 : R=$NHCH_2CH_2OAc$, X=Br

20 : R=OMe, X=I

Chart 3

phenol betaine (**16**) was converted to a phthalimido derivative (**17**). Hydrazinolysis of **17** by a method similar to that used for **2** gave a (2-hydroxyethyl)amino derivative (**18**) quantitatively, as expected. The structure of **18** was confirmed by the following evidence. Acetylation of **18** with acetic anhydride–pyridine at room temperature afforded an O-acetate (**19**) having a carbonyl band at 1740 cm^{-1}. The absorption spectrum of **18** showed bathochromic shifts in comparison with that of papaverine methiodide (**20**), as summarized in Table I. On sodium borohydride reduction, **18** afforded a tetrahydro derivative (**21**) whose spectral data were consistent with the structure **21**. This is the first example of the Smiles rearrangement in an isoquinolinium salt.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were taken in KBr disks with a Hitachi EPI-S2 spectrophotometer. UV and visible spectra were obtained with a Hitachi EPS-2U spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a Varian HA-100 or Varian EM-360A instrument with tetramethylsilane as an internal standard. The following abbreviations are used; s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad. MS were obtained with a Hitachi RMU-6L mass spectrometer. Organic extracts were dried over anhydrous Na_2SO_4, and evaporated *in vacuo*.

5,6-Dihydro-9-hydroxy-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizidine Phenol Betaine (6)——A solution of NaOH (10 g) in H_2O (200 ml) was added to a solution of 9-demethyldehydrocorydalinium chloride (**5**) (10 g, 26 mmol) in H_2O (200 ml) and the resulting precipitate was collected by filtration. The precipitate was extracted with $CHCl_3$. The extract was dried over K_2CO_3 and evaporated. The residue was washed with diethyl ether (ether) and air-dried to give **6** (7.5 g), pale brown crystalline powder, mp > 260 °C (dec.). *Anal.* Calcd for $C_{21}H_{21}NO_4 \cdot H_2O$: C, 68.28; H, 6.28; N, 3.79. Found: C, 68.01; H, 5.66; N, 3.83. UV λ_{max} nm (log ε): 238 (4.47), 279 (4.41), 310 sh (4.05), 384 (4.10), 502 (3.81); λ_{min} nm (log ε): 257 (4.20), 340 (3.76), 441 (3.51).

5,6-Dihydro-2,3,10-trimethoxy-13-methyl-9-(2-phthalimidoethoxy)dibenzo[*a*,*g*]quinolizinium Bromide (2)——A mixture of **6** (2.5 g, 5.3 mmol), 2-bromoethylphthalimide (6.5 g, 26 mmol) and dioxane (100 ml) was heated under reflux for 17 h. Ether was added to the reaction mixture and the resulting precipitate was collected by filtration. The

precipitate was recrystallized from EtOH–ether to give **2** (1.8 g), yellow needles, mp 163—165 °C (dec.). *Anal.* Calcd for $C_{31}H_{29}BrN_2O_6 \cdot H_2O$: C, 59.71; H, 5.01; Br, 12.81; N, 4.49. Found: C, 59.40; H, 4.96; Br, 12.57; N, 4.42. IR (KBr): 1770, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.97 (3H, s, C_{13}-CH_3), 3.25 (2H, m, C_5-H_2), 3.87 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 4.00 (3H, s, OCH_3), 4.30 (2H, m, $CH_2NC{=}O$), 4.78 (2H, m, C_6-H_2), 5.12 (2H, m, OCH_2CH_2), 7.00 (1H, s, C_4-H), 7.21 (1H, s, C_1-H), 7.8—7.95 (6H, m, aromatic protons), 10.28 (1H, s, C_8-H).

5,6-Dihydro-9-(2-hydroxyethyl)amino-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium Bromide (3)——A mixture of **2** (3.1 g, 5 mmol), hydrazine hydrate (100%, 0.8 g, 16 mmol) and EtOH (180 ml) was heated under reflux for 2 h. After removal of the solvent, the residue was acidified with 10% HBr (7 ml) and diluted with a mixture of EtOH–H_2O. The resulting precipitate was removed by filtration and the filtrate was concentrated. The residual solution was extracted with $CHCl_3$ and the solvent was evaporated off. The residue was recrystallized from EtOH–ether to give **3** (2.31 g), yellow prisms, mp 233—236 °C (dec.). *Anal.* Calcd for $C_{23}H_{27}BrN_2O_4 \cdot 0.5H_2O$: C, 57.03; H, 5.83; Br, 16.50; N, 5.78. Found: C, 57.22; H, 5.86; Br, 17.20; N, 5.68. IR (KBr): 3350 (OH), 2960 (NH) cm^{-1}. UV λ_{max} nm (log ε): 232 (4.50), 283 (4.47), 346 (4.17), 480 (3.86); λ_{min} 260 (4.23), 314 (4.05), 406 (3.45). ^{1}H-NMR (DMSO-d_6) δ: 2.89 (3H, s, C_{13}-CH_3), 3.17 (2H, m, C_5-H_2), 3.65 (4H, s, NCH_2CH_2O), 3.87 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 4.01 (3H, s, OCH_3), 4.78 (2H, m, C_6-H), 7.07 (1H, s, C_4-H), 7.19 (1H, s, C_1-H), 7.72 (1H, d, C_{11}-H or C_{12}-H), 7.82 (1H, d, C_{11}-H or C_{12}-H), 10.10 (1H, s, C_8-H).

5,6,7,8,13,13a-Hexahydro-9-(2-hydroxyethyl)amino-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizidine (7)——Sodium borohydride (0.38 g) was added portionwise to a solution of **3** (0.48 g) in MeOH (20 ml) at room temperature and the mixture was allowed to stand for 17 h. After removal of the MeOH, the residue was extracted with $CHCl_3$. The extract was dried and evaporated to give **7** (0.26 g) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, d, J=7.7 Hz, C_{13}-CH_3), 3.87 (9H, s, $OCH_3 \times 3$), 6.62 (1H, s, C_4-H), 6.70 (1H, s, C_1-H), 6.82 (2H, C_{11}-H and C_{12}-H). **7** was treated with ethanolic HCl in ether to give the hydrochloride as colorless needles, mp 195—200 °C. *Anal.* Calcd for $C_{23}H_{30}N_2O_4 \cdot 2HCl \cdot H_2O$: C, 56.44; H, 6.99; Cl, 14.49; N, 5.73. Found: C, 56.36; H, 6.59; Cl, 14.42; N, 5.69. ^{1}H-NMR ($CDCl_3$) δ: 1.15 (3H, d, J=7.5 Hz, C_{13}-CH_3), 3.79 (6H, s, $OCH_3 \times 2$), 3.93 (3H, s, OCH_3), 6.86 (1H, s, C_4-H), 7.00 (1H, s, C_1-H), 7.33 (2H, s, C_{11}-H and C_{12}-H). MS *m/z* (%): 398 (M^+, 14), 353 ($M^+ - 45$, 16), 192 (45), 207 (100), 176 (85).

9-(2-Acetoxyethyl)amino-5,6-dihydro-2,3,10-trimethoxydibenzo[*a*,*g*]quinolizinium Bromide (8)——A mixture of **3** (0.15 g, 0.3 mmol), acetic anhydride (10 ml) and pyridine (25 ml) was allowed to stand at room temperature for 17 h. After removal of the solvent and excess acetic anhydride, the residue was washed with ether and recrystallized from EtOH–ether to give **8** (0.1 g), yellow needles, mp 173—175 °C (dec.). *Anal.* Calcd for $C_{27}H_{31}BrN_2O_6$: C, 57.97; H, 5.59; Br, 14.28; H, 5.01. Found: C, 58.07; H, 5.50; Br, 14.11; N, 5.00. IR (KBr): 3270, 1730 cm^{-1}. UV λ_{max} nm (log ε): 232 (4.52), 283 (4.49), 346 (4.22), 479 (3.85); λ_{min} nm (log ε): 260 (4.24), 314 (4.06), 405 (3.44). ^{1}H-NMR ($CDCl_3$) δ: 2.01 (3H, s, $COCH_3$), 2.90 (3H, s, C_{13}-CH_3), 3.15 (2H, m, C_5-H), 3.97 (6H, s, $OCH_3 \times 2$), 4.00 (3H, s, OCH_3), 4.0 (2H, m, NCH_2), 4.38 (2H, m, CH_2–O–), 5.04 (2H, m, C_6-H), 6.95 (1H, s, C_4-H), 7.22 (1H, s, C_1-H), 7.51 (1H, s, C_{11}-H or C_{12}-H), 7.63 (1H, s, C_{11}-H or C_1-H).

9-[*N*-(2-Acetoxyethyl)-*N*-acetylamino]-5,6-dihydro-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium Bromide (9)——A mixture of **3** (0.2 g, 0.4 mmol) and acetic anhydride (25 ml) was heated at 100 °C for 1.5 h, and then concentrated. The residue was recrystallized from EtOH–ether containing a drop of 10% HBr, to give **9** (0.15 g), yellow prisms, mp 204—207 °C (dec.). *Anal.* Calcd for $C_{27}H_{31}BrN_2O_6$: C, 57.97; H, 5.59; Br, 14.28; N, 5.01. Found: C, 58.07; H, 5.50; Br, 14.11; N, 5.00. UV λ_{max} nm (log ε): 232 (4.46), 263 (4.46), 339 (4.42), 420 (3.72); λ_{min} (log ε): 246 (4.33), 308 (4.10), 378 (3.42). IR (KBr): 1735, 1665 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.01 (3H, s, $OCOCH_3$), 2.90 (3H, s, $NCOCH_3$), 3.97 (6H, s, $OCH_3 \times 2$), 4.00 (3H, s, OCH_3), 4.38 (2H, m, CH_2NAc), 5.04 (2H, m, OCH_2), 6.95 (1H, s, C_8-H), 7.51 (1H, d, J=8 Hz, C_{11}-H or C_{12}-H), 7.63 (1H, d, J=8 Hz, C_{11}-H or C_{12}-H).

9-[*N*-Acetyl-*N*-(2-hydroxyethyl)]amino-5,6-dihydro-2,3,10-trimethoxy-13-methyldibenzo[*a*,*g*]quinolizinium Bromide (10)——The diacetate (**9**) (0.1 g) was repeatedly recrystallized from EtOH to yield **10** (0.03 g), yellow prisms, mp 245—250 °C (dec.). *Anal.* Calcd for $C_{25}H_{29}BrN_2O_5$: C, 58.03; H, 5.64; Br, 15.44; N, 5.41. Found: C, 57.94; H, 5.64; Br, 15.72; N, 5.21. IR (KBr): 1660 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.70 (3H, s, $NCOCH_3$), 3.06 (3H, s, C_{13}-CH_3), 3.88 (3H, s, OCH_3), 7.21 (1H, s, C_4-H), 7.43 (1H, s, C_1-H), 8.30 (1H, d, J=9 Hz, C_{11}-H or C_{12}-H), 8.53 (1H, d, J=9 Hz, C_{11}-H or C_{12}-H).

5,6-Dihydro-10-methoxy-2,3-methylenedioxy-9-(2-phthalimidoethoxy)dibenzo[*a*,*g*]quinolizinium Bromide (11)——A mixture of **13** (0.5 g, 1 mmol), 2-bromoethylphthalimide (1.3 g, 5 mmol), dioxane (20 ml) and dimethylformamide (DMF) (10 ml) was heated under reflux for 5 h. Ether was added to the reaction mixture and the resulting precipitate was collected by filtration. The precipitate was recrystallized from MeOH to give **11** (0.3 g), yellow needles, mp 230—235 °C (dec.). *Anal.* Calcd for $C_{29}H_{23}BrN_2O_6$: C, 60.53; H, 4.03; Br, 13.89. Found: C, 60.44; H, 3.79; Br, 13.64; N, 4.96. IR (KBr): 1770, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.35 (2H, m, C_5-H_2), 4.05 (3H, s, OCH_3), 4.40 (2H, t, CH_2NCO), 4.80 (2H, m, C_6-H_2), 4.95 (2H, m, OCH_2CH_2), 6.15 (2H, s, OCH_2O), 6.94 (1H, s, C_4-H), 7.50 (1H, s, C_1-H), 7.8—8.2 (6H, m, aromatic protons), 8.4 (1H, s, C_{13}-H), 9.80 (1H, s, C_8-H).

9-(2-Hydroxyethyl)amino-2,3-methylenedioxy-10-methoxy-5,6,7,8,13,13a-hexahydrodibenzo[*a*,*g*]quinolizidine (15)——A mixture of **11** (0.1 g, 0.17 mmol), hydrazine hydrate (100%, 0.03 g, 6 mmol) and MeOH (14 ml) was heated under reflux for 3 h. The reaction mixture was acidified with 10% HBr and concentrated *in vacuo*. The residue was

extracted with $CHCl_3$ and the solvent was removed. The residue was treated with EtOH. The EtOH-soluble fraction was concentrated to give crude **14**, which was treated with $NaBH_4$–MeOH as in the case of reduction of **3**, to give crude **15** (0.05 g). Purification of **15** by means of preparative thin-layer chromatography followed by recrystallization from ether gave an analytical sample as colorless prisms, mp 174—175 °C. *Anal.* Calcd for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57; N, 7.60. Found: C, 68.20; H, 6.41; N, 7.35. ^{1}H-NMR ($CDCl_3$) δ: 2.2—3.0 (3H, m), 3.05—3.30 (4H, m), 3.35—3.70 (4H, m), 3.83 (3H, s, OCH_3), 4.20 (1H, d, J=16 Hz, C_8-H_2), 5.90 (2H, s, OCH_2O), 6.57 (1H, s, C_4-H), 6.72 (1H, s, C_1-H), 6.75 (2H, s, C_{11}-H or C_{12}-H). MS *m/z* (%): 368 (M^+, 15), 323 (M^+-45), 323 (32), 193 (100), 178 (40), 162 (50).

7-Methoxy-1-(3,4-dimethoxybenzyl)-2-methyl-6-(2-phthalimidoethoxy)isoquinolinium Bromide (17)——A mixture of **16** (1.5 g, 4.4 mmol), 2-bromoethylphthalimide (5.6 g, 22 mmol), dioxane (20 ml) and DMF (5 ml) was heated under reflux for 20 h. Ether was added to the reaction mixture and the resulting precipitate was collected by filtration. The precipitate was recrystallized from EtOH–EtOAc to give **17** (1.2 g), mp 220—227 °C (dec.). *Anal.* Calcd for $C_{30}H_{29}BrN_2O_6 \cdot 0.5H_2O$: C, 59.81; H, 4.85; Br, 13.26; N, 4.65. Found: C, 59.65; H, 4.73; Br, 13.52, N, 4.80. IR (KBr): 1765, 1700 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.70 (6H, s, $OCH_3 \times 2$), 3.88 (3H, s, OCH_3), 4.15 (2H, br, CH_2–N), 4.34 (3H, s, NCH_3), 4.58 (2H, br, CH_2-Ar), 5.0 (2H, br, OCH_2), 6.45 (1H, dd, J=2, 8 Hz, C_6-H), 6.63 (1H, d, J=8 Hz, $C_{5'}$-H), 7.0 (1H, d, J=2 Hz, $C_{2'}$-H), 7.86 (2H, s, C_5-H and C_8-H), 8.2 (1H, br, C_4-H), 8.5 (1H, br, C_3-H).

6-(2-Hydroxyethyl)amino-7-methoxy-1-(3,4-dimethoxybenzyl)-2-methylisoquinolinium Bromide (18)——According to the same procedure as described above for the preparation of **3**, hydrazinolysis of **17** (0.6 g, 1 mmol) with hydrazine hydrate (100%, 0.16 g, 3 mmol) in EtOH (30 ml) gave **18** (0.42 g), mp 137—140 °C. *Anal.* Calcd for $C_{22}H_{27}BrN_2O_4 \cdot 0.25H_2O$: C, 56.48; H, 5.92; Br, 7.08; N, 5.99. Found: C, 56.62; H, 6.09; Br, 17.20; N, 5.93. ^{1}H-NMR (DMSO-d_6) δ: 3.5 (2H, br, NCH_2CH_2), 3.6 (2H, br, OCH_2CH_2), 3.68 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 4.17 (3H, s, N^+–CH_3), 4.88 (2H, br, CH_2-Ar), 6.45 (1H, dd, J=2, 8 Hz, $C_{6'}$-H), 6.85 (1H, d, J=8 Hz, $C_{5'}$-H), 6.99 (1H, d, J=2 Hz, $C_{2'}$-H), 7.24 (1H, s, C_5-H), 7.55 (1H, s, C_8-H), 7.84 (1H, d, C_4-H), 8.26 (1H, s, C_3-H).

6-(2-Acetoxyethyl)amino-7-methoxy-1-(3,4-dimethoxybenzyl)-2-methylisoquinolinium Bromide (19)——A mixture of **18** (0.1 g, 2.1 mmol), acetic anhydride (10 ml) and pyridine (10 ml) was allowed to stand for 17 h. After removal of the solvent and excess acetic anhydride *in vacuo*, the residue was washed with ether and recrystallized from EtOH–ether to give **19** (0.07 g), pale yellow prisms, mp 155—160 °C. *Anal.* Calcd for $C_{24}H_{29}BrN_2O_5 \cdot H_2O$: C, 55.07; H, 5.97; Br, 15.27; N, 5.35. Found: C, 54.81; H, 5.76; Br, 14.90; N, 5.43. ^{1}H-NMR ($CDCl_3$) δ: 2.10 (3H, s, $OCOCH_3$), 3.80 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 4.05 (3H, s, OCH_3), 4.40 (3H, s, N^+–CH_3), 5.10 (1H, s, NH), 6.10 (1H, br, OH), 6.30 (1H, dd, $C_{6'}$-H), 6.75 (1H, d, $C_{5'}$-H), 6.95 (2H, s, $C_{2'}$-H), 7.30 (1H, s, C_5-H or C_8-H), 7.40 (1H, s, C_5-H or C_8-H), 7.80 (1H, d, C_4-H), 8.50 (1H, d, C_3-H).

1,2,3,4-Tetrahydro-6-(2-hydroxyethyl)amino-7-methoxy-1-(3,4-dimethoxybenzyl)-2-methylisoquinoline (21)——According to the same procedure as described above for the preparation of **7**, reduction of **18** (0.1 g) with $NaBH_4$–MeOH gave **21** (0.04 g), colorless prisms, mp 125—127 °C. *Anal.* Calcd for $C_{22}H_{30}N_2O_4$: C, 68.37; H, 7.82; N, 7.25. Found: C, 68.10; H, 7.55; N, 7.42. MS *m/z* (%): 386 (M^+, 2), 236 (100), 221 (6), 204 (8), 190 (29). ^{1}H-NMR ($CDCl_3$) δ: 2.55 (3H, s, N–CH_3), 3.55 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.60 (1H, s, C_5-H), 6.58 (1H, s, C_8-H), 6.60—6.90 (3H, m, aromatic protons).

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Studies on Bi-heterocyclic Compounds. I. 6-Substituted Dihydro-1,4-thiazinones[1)]

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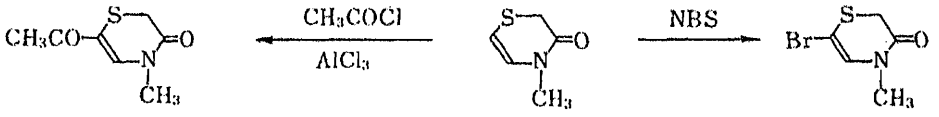
Reactions of 5-methyl-2*H*-1,4-thiazin-3(4*H*)-one (**4**) with various *N*-acylpyridinium salts (**7a**—**g**) led to (*N*-acyldihydropyridyl)thiazinones (**5a**—**g**), oxidation of which yielded a new class of pyridylthiazinones (**6a**—**g**). These reactions were applied to the synthesis of other azaarylthiazinones. Some of these azaarylthiazinones, particularly 6-(4-pyridyl)thiazinones (**6a**, **14a** and **14b**) showed positive inotropic activity with little chronotropic effect on guinea pig left atria.

Keywords——1,4-thiazine; dihydropyridine; dihydropyridyl-1,4-thiazinone; pyridyl-1,4-thiazinone; *N*-acylpyridinium salt; oxidation; positive inotropic activity; congestive heart failure; bi-heterocyclic compound

Some chemical reactions of dihydro-1,4-thiazinones such as **1** are known,[2)] but the number of examples is relatively few. Recently, Hojo *et al.* reported that the *N*-methyl-1,4-thiazinone (**1**) undergoes electrophilic attack at the 6-position; for example, Friedel–Crafts acylation[3)] or bromination[4)] of **1** gave the 6-acetyl (**2**) or 6-bromo derivative (**3**), respectively.



2 1 3

Chart 1

During our extensive synthetic studies on dihydro-1,4-thiazinones, it was found that 5-methyl-2*H*-1,4-thiazin-3(4*H*)-one (**4**) undergoes electrophilic substitution reactions at the 6-position just as well, even though the nitrogen atom is not substituted. We report here the substitution reaction with several *N*-acylpyridinium salts to form the corresponding 6-(1,4-dihydropyridyl)thiazinones (**5a**—**g**), and the oxidative transformation of **5a**—**g** to the 6-pyridylthiazinones (**6a**—**g**). Some of the 6-pyridylthiazinones thus obtained showed a potent positive inotropic activity with little chronotropic effect on guinea pig atria.

5-Methyl-2*H*-1,4-thiazin-3(4*H*)-one (**4**) was prepared by the method of Rao *et al.*[5)] and treated with 2,2,2-trichloroethyl chloroformate in the presence of pyridine in acetonitrile, affording the substituted thiazinone (**5a**) in 81% yield. The infrared (IR) spectrum of **5a** exhibited absorptions at 1720 and 1670 cm^{-1}, indicating the presence of urethane and amide carbonyl moieties, respectively. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **5a** showed singlets at δ 1.99 due to the 5-methyl protons and at δ 3.23 due to the 2-methylene protons of the thiazinone skeleton. In addition, disappearance of the signal due to

the 6-proton (6-H) at δ 5.25 in **4** suggests the existence of a 6-substituent in **5a**. Further, doublet at δ 6.97 with a coupling constant (J) of 7 Hz may be ascribed to H-2 and H-6 of an α,β-unsubstituted dihydropyridine ring, reflecting the 4-substituted 1,4-dihydropyridine structure of **5a**. The signals of H-3 and H-5 of the dihydropyridine ring appear as a multiplet at δ 4.80, which overlaps with signals due to the methylene protons of the trichloroethyl group, while the γ-proton signal appears as a multiplet at δ 4.16.

No isomeric 1,2-dihydro-2-pyridyl compound (**8**) was detected in the reaction product, suggesting that this reaction proceeds regioselectively.

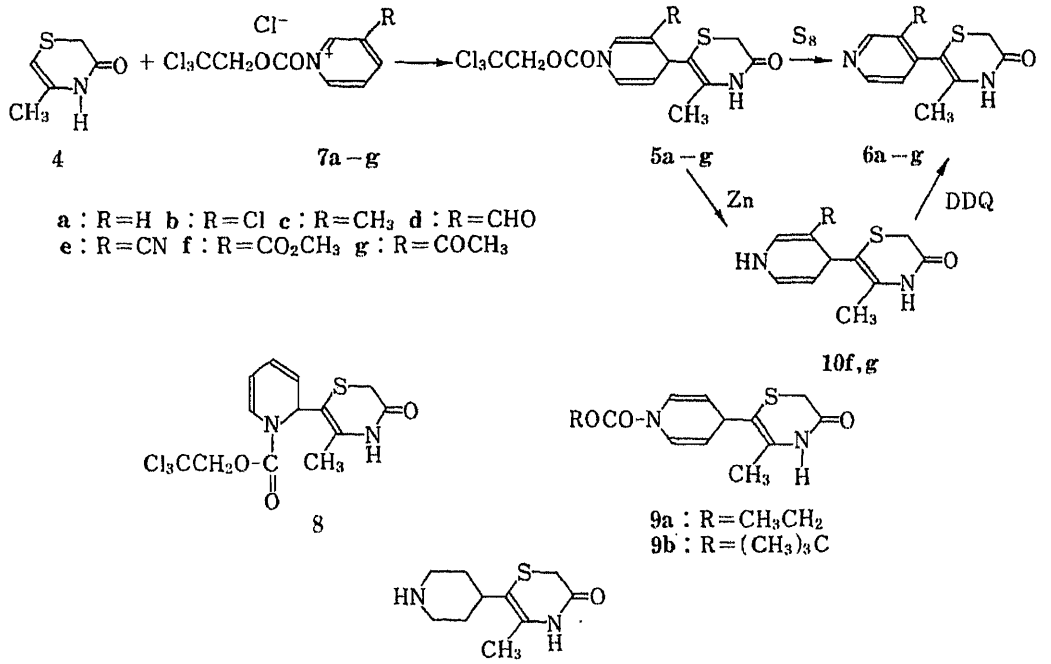
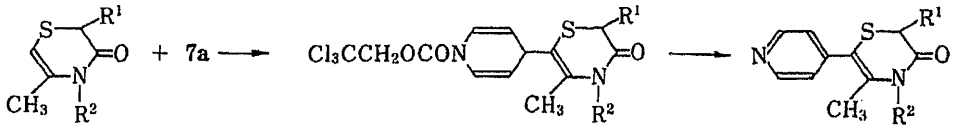


Chart 2

The 1-ethoxycarbonyl and 1-*tert*-butoxycarbonyl salts were also found to react with **4** to give the corresponding analogs of **5a**, but the yield was not satisfactory.

The dihydropyridine compound (**5a**) was readily oxidized with sulfur at 140 °C to provide the 6-pyridylthiazinone (**6a**) in good yield. The structure of **6a** was assigned on the basis of spectral evidence. The IR spectrum of **6a** lacked the absorption due to the urethane group. The presence of a 4-substituted pyridine ring in **6a** was shown by its NMR signals at δ 7.30 (dd, J=1.5, 5 Hz, H-3 and H-5) and δ 8.60 (d, J=5 Hz, H-2 and H-6).

When using 3-substituted pyridinium salts, including the 3-chloro, 3-methyl, 3-formyl, 3-cyano, 3-methoxycarbonyl and 3-acetyl derivatives (**7b—g**), reactions with the thiazinone (**4**) proceeded similarly, giving the corresponding 3-substituted 6-[1-(2,2,2-trichloroethoxycarbonyl)-1,4-dihydro-4-pyridyl]thiazinones (**5b—g**). Oxidation of these dihydropyridine derivatives (**5—e**) with sulfur provided the corresponding 3-substituted 6-pyridylthiazinones (**6b—e**), while the 3-methoxycarbonyl and 3-acetyl derivatives (**5f** and **5g**) gave only a complex tar under the same conditions. Treatment of **5f** and **5g** with zinc powder in aqueous tetrahydrofuran under acidic conditions (pH 4—5) afforded dihydropyridylthiazinones (**10f** and **10g**, respectively). The structures of **10f** and **10g** were confirmed by the appearance of secondary amine absorptions at 3300 cm^{-1} in the IR spectra and the disappearance of the urethane absorption. The dihydropyridine compounds (**10f** and **10g**) were readily oxidized by treatment with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in acetonitrile



12a–c 13a–c 14a–c

a : $R^1=CH_3$, $R^2=H$ b : $R^1=CH_3CH_2$, $R^2=H$ c : $R^1=H$, $R^2=CH_3$

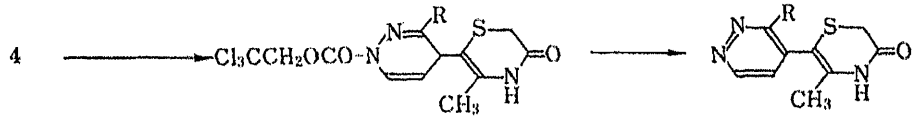
Chart 3

to give 6-pyridylthiazinones (**6f** and **6g**, respectively). On the other hand, treatment of the unsubstituted dihydropyridine (**5a**) with zinc powder in formic acid failed to afford the *N*-deacylated dihydro-compound, but gave 6-(4-piperidyl)thiazinone (**11**) along with a small amount of 6-pyridylthiazinone (**6a**).

2,5-Dimethyl- and 2-ethyl-5-methylthiazinones (**12a** and **12b**) were obtained by the same method as reported for the synthesis of **4**[5] (see Experimental). Treamtent of **12a** and **12b** with the pyridinium salt **7a** followed by oxidation of the resulting 7-dihydropyridylthiazinones (**13a** and **13b**) with sulfur afforded the 2-substituted 4-pyridylthiazinones (**14a** and **14b**, respectively) in good yields. The 4,5-dimethylthiazinone (**12c**), obtained by reaction of **4** with methyl iodide in the presence of sodium hydride,[6] was also treated with **7a** to provide the dihydropyridylthiazinone (**13c**), but in a lower yield. Treatment of **13c** with sulfur gave the pyridylthiazinone (**14c**).

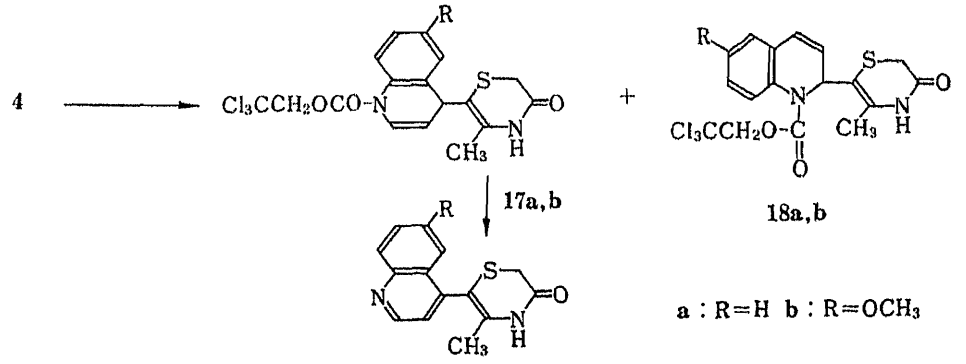
Next, we examined the substitution reactions of the thiazinone (**4**) with salts derived from other heterocycles such as pyridazine, quinoline and thiazole.

Treatment of **4** with pyridazine in the presence of 2,2,2-trichloroethyl chloroformate gave the dihydropyridazinylthiazinone (**15a**), oxidation of which with sulfur provided the 6-pyridazinylthiazinone (**16a**). The NMR spectrum of **16a** exhibited a doublet of doublets at δ 7.65 assignable to H-5 of the pyridazine ring coupled to a doublet at δ 9.20 due to H-6 (J=



15a,b 16a,b

a : R=H b : R=CH_3



18a,b

a : R=H b : R=OCH_3

19a,b

Chart 4

5 Hz) and a doublet at δ 9.25 due to H-3 (J=2.4 Hz), indicating a presence of a 4-substituted pyridazine ring. Reaction of **4** with a salt derived from 3-methylpyridazine gave **15b**. Oxidation of **15b** yielded the 6-pyridazinylthiazinone (**16b**).

Reaction of **4** with quinoline in the presence of 2,2,2-trichloroethyl chloroformate gave a mixture of (1,4-dihydro-4-quinolyl)thiazinone (**17a**) and (1,2-dihydro-2-quinolyl)thiazinone (**18a**) in a ratio of 4 : 1. The NMR spectrum of **17a** exhibited a spectral pattern similar to those of (1,4-dihydro-4-pyridyl)thiazinones (**5a—g**): a doublet at δ 4.62 due to H-4 of the quinoline ring, a doublet of doublets at δ 5.20 due to H-3 and a doublet at δ 7.21 due to H-2 with $J_{2,3}$ of 8 Hz and $J_{3,4}$ of 4 Hz, suggesting a 4-substituted 1,4-dihydroquinoline structure for **17a**. Further, oxidation of **17a** gave 4-quinolylthiazinone (**19a**), whose NMR spectrum showed a doublet at δ 7.35 due to H-3 and a doublet at δ 8.95 due to H-2 with $J_{2,3}$ of 4.4 Hz, confirming the existence of the 4-substituted quinoline structure for **19a**. The NMR spectrum of the isomeric dihydroquinolylthiazinone (**18a**) differed from that of **17a**, exhibiting a marked upfield shift of a doublet at δ 6.13 with J of 6 Hz in comparison with that (δ 4.62) in **17a**. Considering the electronic effects of the neighboring nitrogen atom, the doublet at δ 6.13 may be assignable to H-2, suggesting the 2-substituted 1,2-dihydroquinoline structure for **18a**. Oxidation of **18a** with sulfur gave a complex tar, from which the desired quinoline product could not be obtained.

Reaction of the thiazinone (**4**) with a 6-methoxyquinolinium salt proceeded analogously to give the 4-substituted compound (**17b**) and the 2-substituted compound (**18b**) in a ratio of 1 : 1. Oxidation of **17b** with sulfur gave the 6-(4-quinolyl)thiazinone (**19b**).

Some electrophilic properties of thiazolium salts are well knwon.[7] Similar reactions of the thiazinone (**4**) with thiazole in the presence of ethyl chloroformate or 2,2,2-trichloroethyl chloroformate were carried out to give the corresponding 1-acyldihydro-(2-thiazolyl)-thiazinones, (**20a** or **20b**). Their structures were confirmed by NMR spectrometry. A similar conversion of **20b** to thiazolylthiazinone with sulfur or DDQ was unsuccessful, and unchanged **20b** was recovered. Treatment of **20a** with DDQ in dichloromethane afforded the desired (2-thiazolyl)thiazinone (**21**) in good yield.

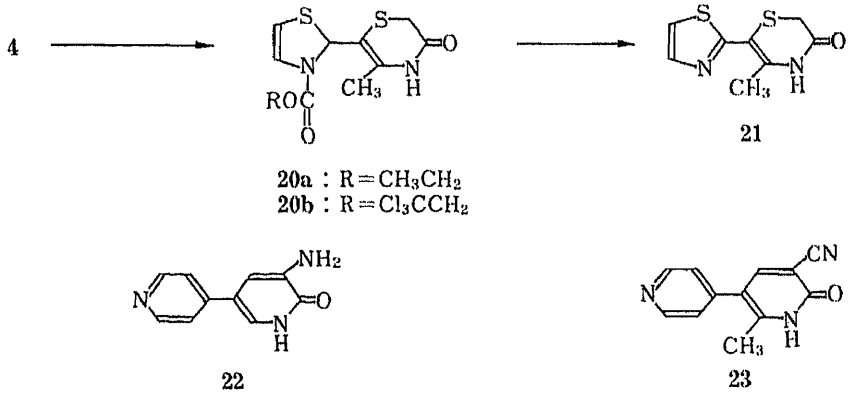


Chart 5

Biological Results

Much attention has recently been focused on the development of new cardiotonic agents, because there is a need for less toxic drugs than cardiac glycosides, which remain the basic agents for treatment of congestive heart failure.

Pyridylpyridine compounds such as amrinone (**22**) and milrinone (**23**)[8] have recently been developed as new cardiotonic agents, and their clinical usefulness has been proved. The structural similarity of these bi-heterocyclic compounds to the compounds described above

TABLE I. Effects of Thiazinone Derivatives on Developed Tension on the Guinea Pig Left Atrium[a]

Compd.[c]	Change in developed tension[b] (mg)
6a	511 ± 70
6b	426 ± 56
6c	278 ± 56
6d	194 ± 21
6e	214 ± 56
6f	0
6g	75 ± 21
11	0
14a	588 ± 30
14b	582 ± 44
14c	100 ± 50
16a	225 ± 123
16b	111 ± 34
19a	300 ± 125
19b	103 ± 41
21	97 ± 16

a) Suspended in Krebs-Henseliet solution bubbled with 95% O_2 and 5% CO_2 at 30°C. *b*) The left atrium, whose resting tension was adjusted to 500 mg, was stimulated by square pulses of 5-ms duration, a voltage of 20% above the threshold and a stimulating rate of 0.5 Hz. Contractive forces were measured as absolute changes in developed tension. *c*) Concentration, 10^{-4} M in water, except for **16a** and **19a** (10^{-5} M).

prompted us to study their pharmacological activity, particularly in regard to cardiotonic activity.

We tested these compounds primarily for inotropic activity using the isolated left atria of guinea pig. The results of this screening are given in Table I. The most active compound was the unsubstituted pyridylthiazinone (**6a**) and the introduction of substituents into the pyridine ring of **6a** led to less active derivatives. Replacement of the pyridine ring of **6a** by pyridazine, quinoline or thiazole reduced the activity. Introduction of lower alkyl groups into the 2-position of the thiazinone ring as in **14a** and **14b** resulted in unchanged or somewhat enhanced activity. Piperazinylthiazinone (**11**) and pyridyl-4-methylthiazinone (**14c**) showed no activity.

Experimental

Melting points were determined on a Yamato MP-1 apparatus, and are uncorrected. NMR spectra were recorded on a JEOL FX-270 spectrometer with tetramethylsilane as the reference, and IR spectra were recorded on a Hitachi 260-10 spectrometer. The results of detailed characterization (yields, elemental analyses, IR, NMR spectra) of the bi-heterocyclic compounds reported here are summarized in Tables II and III. Thin-layer chromatography (TLC) was performed on TLC plates, Silica gel $60F_{254}$ precoated, layer thickness 0.2 mm (E. Merck) and spots were detected under ultraviolet (UV) irradiation. Column chromatography was done on Wakogel C-200 and the developing solvents are shown in parentheses.

Thiazinones (4 and 12a—c)——5-Methyl-2*H*-1,4-thiazin-3(4*H*)-ones (**4**) were prepared by a procedure developed by Rao *et al.*[5] The 4-methyl derivative of **4** (**12c**) was synthesized from **4** by the method of deStevens *et al.*[6] Preparation of 2,5-dimethyl-2*H*-1,4-thiazin-3(4*H*)-one (**12a**) was carried out by a modification of the method of Rao *et al.*[5] as described below.

A mixture of ethyl α-mercaptopropionate (19.0 g, 141 mmol) and 28% ammonia (100 ml) was stirred at room temperature for 20 h under an N_2 atmosphere and then evaporated to dryness *in vacuo*, to give α-mercaptopropionamide as a colorless solid. A solution of monochloroacetone (13.9 g, 150.7 mmol) in Et_2O (40 ml) was added dropwise to a mixture of α-mercaptopropionamide and triethylamine (14.5 g, 143.4 mmol) in absolute EtOH over a period of 2 h at 0°C with stirring, and the mixture was stirred at room temperature for 2 h, then evaporated at 50°C *in vacuo*. The residue was dissolved in absolute EtOH (100 ml) and the solution was acidified to pH 1—2 by adding *p*-toluenesulfonic acid monohydrate portionwise with shaking. The mixture was stirred at 60—70°C for 30 min and

TABLE II. Data for (1,4-Dihydro-4-pyridinyl)thiazinones and 1-Alkoxy Derivatives

Compd.	mp (°C) Recrystn. solvent	IR ν_{max}^{KBr} cm^{-1}	NMR ($CDCl_3$) δ [b]	Analysis (%) Calcd (Found)			Formula	Yield (%)
				C	H	N		
5a	158—160 (EtOH)	3200, 3100, 1720, 1670, 1630	1.99 (3H, s), 3.23 (2H, s), 4.16 (1H, m), 4.74—4.93 (2H+2H, m), 6.97 (2H, d, J=7 Hz), 7.26 (1H, br)	40.69 (40.62	3.41 3.37	7.29 7.02)	$C_{13}H_{13}Cl_3N_2O_3S$	81
5b	168.5—170 (EtOH)	3190, 3080, 1720, 1670, 1630	2.07 (3H, s), 3.28 (2H, s), 4.39 (1H, d, J=4 Hz), 4.75—5.06 (2H+1H, s+m), 7.10 (1H, d, J=8 Hz), 7.30 (1H, s), 8.38 (1H, s)	37.34 (37.12	2.89 2.78	6.70 6.67)	$C_{13}H_{12}Cl_4N_2O_3S$	66
5c	152—154 (AcOEt)	3230, 3120, 1735, 1690, 1650	1.65 (3H, s), 2.03 (3H, s), 3.21 (2H, ABq, J=14.6 Hz), 4.02 (1H, d, J=3 Hz), 4.74—4.96 (2H+1H, m), 6.80 (1H, d, J=6 Hz), 6.98 (1H, dd, J=6, 8 Hz), 8.04 (1H, s)	42.28 (42.07	3.80 3.77	7.04 6.81)	$C_{14}H_{15}Cl_3N_2O_3S$	32
5d	154—156 (EtOH)	3290, 3120, 1745, 1680, 1620	2.17 (3H, s), 3.14 (2H, s), 4.53 (1H, d, J=5 Hz), 4.99 (2H, s), 5.14 (1H, dd, J=5, 8 Hz), 7.13 (1H, dd, J=2, 8 Hz), 7.91 (1H, d, J=2 Hz), 8.23 (1H, s), 9.53 (1H, s)	40.84 (40.65	3.18 3.24	6.80 6.68)	$C_{14}H_{13}Cl_3N_2O_4S$	44
5e	188.5—190.5 (dec.) (EtOH)	3220, 3120, 2240, 1730, 1680, 1640	2.08 (3H, s), 3.30 (2H, ABq, J=15 Hz), 4.36 (1H, d, J=4.6 Hz), 4.78—5.02 (2H+1H, m), 7.00 (1H, d, J=8 Hz), 7.62 (1H, s), 7.84 (1H, s)	41.14 (41.05	2.95 2.91	10.28 10.10)	$C_{14}H_{12}Cl_3N_3O_3S$	63
5f	170—172 (EtOH)	3350, 3300, 1730, 1670, 1610	2.10 (3H, s), 3.16 (2H, s), 3.76 (3H, s), 4.47 (1H, d, J=4.4 Hz), 4.76—5.10 (2H+1H, m), 7.00 (1H, d, J=8 Hz), 7.82 (1H, s), 8.09 (1H, s)	40.78 (40.52	3.42 3.35	6.34 6.18)	$C_{15}H_{15}Cl_3N_2O_5S$	48
5g	141—142.5 (EtOH)	3200, 3060, 1730, 1660	2.13 (3H, s), 2.34 (3H, s), 3.12 (2H, ABq, J=14.6 Hz), 4.53 (1H, d, J=4.6 Hz), 4.80—5.13 (2H+1H, m), 7.00 (1H, d, J=8 Hz), 7.60 (1H, s), 8.03 (1H, s)	42.31 (42.08	3.55 3.59	6.58 6.37)	$C_{15}H_{15}Cl_3N_2O_4S$	46
13a	140—142 (column)	3200, 3080, 1720, 1670, 1630	1.40 (3H, d, J=7 Hz), 1.98 (3H, s), 3.33 (1H, q, J=7 Hz), 4.16 (1H, m), 4.70—4.94 (2H+2H, m), 6.95 (2H, d×2, J=8 Hz), 7.75 (1H, s)	42.28 (42.33	3.80 3.89	7.04 7.17)	$C_{14}H_{15}Cl_3N_2O_3S$	42
13b	121—122 (column)	3200, 3070, 1720, 1670, 1630	1.00 (3H, t, J=7 Hz), 1.66 (2H, m), 2.00 (3H, s), 3.10 (1H, dd, J=6, 8 Hz), 4.15 (1H, m), 4.60—5.00 (2H+2H, m+s), 7.05 (2H, d, J=8 Hz), 8.80 (1H, s)	43.75 (43.59	4.16 4.23	6.80 6.83)	$C_{15}H_{17}Cl_3N_2O_3S$	27

13c	Oil	1720, 1690, 1660[a)]	2.10 (3H, s), 3.28 (3H, s), 3.41 (2H, s), 4.16 (1H, m), 4.75—4.94 (2H+2H, m), 6.98 (2H, d, J=8.8 Hz)				$C_{14}H_{15}Cl_3N_2O_3S$	33
15a	156—157· (MeOH)	3200, 3090, 1740, 1690	2.03 (3H, s), 3.25 (2H, ABq, J=15 Hz), 4.06 (1H, m), 4.85—5.00 (2H+1H, m), 6.83 (1H, m), 7.22 (1H, d, J=8 Hz), 8.44 (1H, s)	37.46 (37.21	3.14 3.08	10.92 10.80)	$C_{12}H_{12}Cl_3N_3O_3S$	63
15b	200—203 ($CHCl_3$–MeOH)	3200, 3070, 1720, 1670, 1635	1.92 (3H, s), 1.98 (3H, s), 3.17 (2H, ABq, J=15 Hz), 4.20 (1H, d, J=4 Hz), 4.94 (1H, dd, J=4, 8 Hz), 5.03 (2H, s), 7.17 (1H, d, J=8 Hz), 9.64 (1H, s)[c)]	39.16 (39.27	3.53 3.60	10.54 10.73)	$C_{13}H_{14}Cl_3N_3O_3S$	26
17a	168—170 (EtOH)	3200, 3060, 1710, 1660, 1630	2.13 (3H, s), 3.12 (2H, ABq, J=14.4 Hz), 4.62 (1H, d, J=4 Hz), 4.93 (2H, s), 5.20 (1H, dd, J=4, 8 Hz), 7.09—7.33 (3H, m), 7.21 (1H, d, J=8 Hz), 7.90 (1H, s), 8.10 (1H, d, J=8.8 Hz)	47.07 (46.79	3.48 3.45	6.46 6.38)	$C_{17}H_{15}Cl_3N_2O_3S$	24
17b	181.5—183 (EtOH)	3200, 3050, 1720, 1670	2.13 (3H, s), 3.12 (2H, ABq, J=15 Hz), 3.79 (3H, s), 4.61 (1H, d, J=4 Hz), 4.92 (2H, s), 5.16 (1H, dd, J=4, 8 Hz), 6.61 (1H, d, J=3 Hz), 6.84 (1H, dd, J=3, 9 Hz), 7.20 (1H, d, J=8 Hz), 8.04 (1H, d, J=9 Hz), 8.06 (1H, s)	46.61 (46.38	3.69 3.71	6.04 5.94)	$C_{18}H_{17}Cl_3N_2O_4S$	32
18a	199.5—200 (EtOH)	3200, 3100, 1730, 1680, 1640	2.22 (3H, s), 3.02 (2H, s), 4.93 (2H, ABq, J=12 Hz), 5.83 (1H, dd, J=6, 9 Hz), 6.13 (1H, d, J=6 Hz), 6.66 (1H, d, J=9 Hz), 7.03—7.85 (4H, m), 9.51 (1H, s)	47.07 (46.63	3.48 3.49	6.46 6.36)	$C_{17}H_{15}Cl_3N_2O_3S$	6
18b	179—181 (EtOH)	3180, 3070, 1700, 1670, 1630	2.23 (3H, s), 3.03 (2H, s), 3.86 (3H, s), 4.90 (2H, ABq, J=12 Hz), 5.94 (1H, dd, J=6, 9 Hz), 6.16 (1H, d, J=6 Hz), 6.66 (1H, d, J=9 Hz), 6.76 (1H, d, J=3 Hz), 6.93 (1H, dd, J=3, 9 Hz), 7.64 (1H, d, J=9 Hz), 8.57 (1H, s)	46.61 (46.25	3.69 3.54	6.04 5.94)	$C_{18}H_{17}Cl_3N_2O_4S$	26
20a	146—147 ($EtOH–H_2O$)	3220, 3130, 1700, 1680, 1640, 1600	1.30 (3H, t, J=7 Hz), 2.13 (3H, s), 3.25 (2H, s), 4.25 (2H, q, J=7 Hz), 5.62 (1H, d, J=5 Hz), 6.53 (1H, d, J=5 Hz), 6.62 (1H, s), 8.57 (1H, s)	46.13 (45.97	4.92 4.98	9.78 9.74)	$C_{11}H_{14}N_2O_3S_2$	24
20b	141—143 (dec.) ($EtOH–H_2O$)	3200, 3100, 1740, 1680, 1630, 1600	2.14 (3H, s), 3.21 (2H, s), 4.80 (2H, s), 5.67 (1H, d, J=4.4 Hz), 6.55 (1H, d, J=4.4 Hz), 6.61 (1H, s), 8.10 (1H, s)	33.90 (33.75	2.84 2.80	7.18 7.15)	$C_{11}H_{11}Cl_3N_2O_3S_2$	34

a) Measured as liquid. *b)* Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses. *c)* Measured as a solution in DMSO-d_6.

TABLE III. Data for 6-Substituted Thiazinones

Compd.	mp (°C). Recrystn. solvent	IR ν_{max}^{KBr} cm^{-1}	NMR ($CDCl_3$) δ [a]	Analysis (%) Calcd (Found) C	H	N	Formula	Yield (%)
6a	187—188.5 (DMF–H_2O)	3200, 3050, 1680, 1580	2.06 (3H, s), 3.44 (2H, s), 7.30 (2H, dd, J=1.5, 5 Hz), 8.60 (2H, d, J=5 Hz), 8.70 (1H, s)	58.22 (58.48	4.88 4.99	13.58 13.53)	$C_{10}H_{10}N_2OS$	77
6b	169—171 (EtOH–H_2O)	3200, 3080, 1630, 1570	1.85 (3H, s), 3.49 (2H, s), 7.30 (1H, d, J=5 Hz), 8.59 (1H, d, J=5 Hz), 8.76 (1H, s), 9.00 (1H, s)	49.89 (49.68	3.76 3.76	11.64 11.65)	$C_{10}H_9ClN_2OS$	58
6c	185—187 (EtOH)	3040, 1670, 1630, 1590	1.78 (3H, s), 2.31 (3H, s), 3.44 (2H, s), 7.09 (1H, d, J=5 Hz), 8.44 (1H, d, J=5 Hz), 8.51 (1H, s), 8.58 (1H, s)	59.97 (59.62	5.49 5.50	12.71 12.51)	$C_{11}H_{12}N_2OS$	85
6d	152—154 (CH_3CN)	3180, 3060, 1660, 1620	1.85 (3H, s), 3.52 (2H, s), 7.32 (1H, d, J=5.4 Hz), 8.45 (1H, s), 8.80 (1H, d, J=5.4 Hz), 9.11 (1H, s), 10.23 (1H, s)	56.39 (56.43	4.30 4.31	11.95 11.85)	$C_{11}H_{10}N_2O_2S$	10
6e	140—141 (EtOH)	3170, 3050, 2230, 1680, 1600	1.95 (3H, s), 3.50 (2H, s), 7.38 (1H, d, J=5.4 Hz), 8.65 (1H, s), 8.90 (1H, d, J=5.4 Hz), 8.93 (1H, s)	57.12 (57.17	3.92 3.89	18.17 18.14)	$C_{11}H_9N_3OS$	35
6f	177—178 (EtOH)	3300, 1710, 1680, 1620	1.83 (3H, s), 3.46 (2H, s), 3.94 (3H, s), 7.21 (1H, d, J=5.4 Hz), 8.33 (1H, s), 8.72 (1H, d, J=5.4 Hz), 9.12 (1H, s)	54.53 (54.34	4.57 4.51	10.60 10.39)	$C_{12}H_{12}N_2O_3S$	40
6g	160—161.5 (ab. EtOH)	3200, 1680, 1620	1.84 (3H, s), 2.61 (3H, s), 3.43 (2H, s), 7.23 (1H, d, J=5 Hz), 8.27 (1H, s), 8.69 (1H, d, J=5 Hz), 8.88 (1H, s)	58.04 (57.90	4.87 4.69	11.28 11.01)	$C_{12}H_{12}N_2O_2S$	10
11	180—195 (dec.)	3300, 3200, 3050, 1680, 1640	1.50—1.75 (5H, m), 1.97 (3H, s), 2.52—2.70 (3H, m), 3.11—3.17 (2H, m), 3.17 (2H, s), 8.25 (1H, s)	56.56 (57.05	7.59 7.40	13.19 13.10)	$C_{10}H_{16}N_2OS$	36
14a	144—146 (column)	3200, 3090, 1680, 1620	1.53 (3H, d, J=7 Hz), 2.05 (3H, s), 3.53 (1H, q, J=7 Hz), 7.28 (2H, dd, J=1.6, 4.6 Hz), 7.40 (1H, s), 8.61 (2H, d, J=4.6 Hz)	59.97 (59.76	5.49 5.55	12.71 12.55)	$C_{11}H_{12}N_2OS$	52
14b	122—124 (EtOH–H_2O)	3210, 3100, 1670	1.14 (3H, t, J=7.3 Hz), 1.76 (1H, m), 1.99 (1H, m), 2.03 (3H, s), 3.28 (1H, dd, J=6, 9 Hz), 7.27 (2H, dd, J=1.7, 5 Hz), 8.52 (1H, s), 8.61 (2H, d, J=5 Hz)	61.50 (61.63	6.02 6.05	11.95 11.59)	$C_{12}H_{14}N_2OS$	21
14c	139—140 (column)	1660, 1600	2.10 (3H, s), 3.28 (3H, s), 3.41 (2H, s), 7.32 (2H, dd, J=1.5, 5 Hz), 8.62 (2H, d, J=5 Hz)	59.97 (59.70	5.49 5.35	12.71 12.40)	$C_{11}H_{12}N_2OS$	14
16a	231—233 (MeOH)	3200, 3050, 1670, 1600	1.99 (3H, s), 3.46 (2H, s), 7.65 (1H, dd, J=2.4, 5 Hz), 9.20 (1H, dd, J=1, 5 Hz), 9.25 (1H, dd, J=1, 2.4 Hz), 10.18 (1H, s)[b]	52.15 (51.93	4.37 4.27	20.27 20.05)	$C_9H_9N_3OS$	23
16b	187—188 (ab. EtOH)	3200, 3050, 1670, 1620	1.81 (3H, s), 2.75 (3H, s), 3.46 (2H, s), 7.26 (1H, d, J=5 Hz), 8.24 (1H, s), 9.10 (1H, d, J=5 Hz)	54.27 (54.14	5.01 4.76	18.99 18.72)	$C_{10}H_{11}N_3OS$	36
19a	173.5—174 (MeOH)	3200, 3100, 1680, 1640	1.77 (3H, s), 3.56 (2H, s), 7.35 (1H, d, J=4.4 Hz), 7.60 (1H, dd, J=7.8, 8.3 Hz), 7.75 (1H, dd, J=7.8, 8.3 Hz), 7.94 (1H, d, J=8.3 Hz), 7.95 (1H, s), 8.15 (1H, d, J=8.3 Hz), 8.95 (1H, d, J=4.4 Hz)	65.59 (65.45	4.71 4.63	10.93 10.82)	$C_{14}H_{12}N_2OS$	25
19b	183—184 (EtOH)	3200, 3100, 1680	1.82 (3H, s), 3.55 (2H, ABq, J=15 Hz), 3.94 (3H, s), 7.15 (1H, d, J=3 Hz), 7.28 (1H, d, J=4.4 Hz), 7.41 (1H, dd, J=3, 9 Hz), 8.06 (1H, d, J=9 Hz), 8.31 (1H, s), 8.78 (1H, d, J=4.4 Hz)	62.91 (62.90	4.92 4.76	9.78 9.57)	$C_{15}H_{14}N_2O_2S$	32
21	233—235 (dec.) (MeOH)	3200, 3080, 1680, 1615	2.38 (3H, s), 3.43 (2H, s), 7.72 (1H, d, J=3.4 Hz), 7.86 (1H, d, J=3.4 Hz), 10.15 (1H, s)[b]	45.26 (45.28	3.79 3.85	13.19 13.25)	$C_8H_8N_2OS_2$	54

a) Chemical shifts are given with proton numbers, absorption patterns and coupling constants in parentheses. *b*) Measured as a solution in DMSO-d_6.

then evaporated to dryness *in vacuo*. The residue was chromatographed on silica gel (ethyl acetate–hexane = 1 : 1) to give **12a** (3.9 g, 19.2%) as a colorless powder, mp 78—80 °C. NMR ($CDCl_3$) δ: 1.44 (3H, d, J = 7 Hz), 1.95 (3H, s), 3.35 (1H, q, J = 7 Hz), 5.21 (1H, s), 8.07 (1H, s). IR ν_{max}^{KBr} cm^{-1}: 3200, 3100, 1670, 1630. *Anal.* Calcd for C_6H_9NOS: C, 50.32; H, 6.33; N, 9.78. Found: C, 50.06; H, 6.47; N, 9.62.

The synthesis of 2-ethyl-5-methyl-2*H*-1,4-thiazin-3(4*H*)-one (**12b**) was carried out as follows. α-Bromo-*n*-butyryl bromide (18.4 g, 80.2 mmol) was added dropwise to vigorously stirred 28% ammonia (33 ml) at −10 °C and the resulting precipitates were collected by filtration and washed with water to give α-bromo-*n*-butyramide (9.57 g, 72%) as a colorless powder. A mixture of α-bromo-*n*-butyramide (1.66 g, 10.0 mmol) and potassium ethylxanthate (1.6 g, 10.0 mmol) in acetone (20 ml) was stirred at room temperature for 2 h.

The insoluble material was filtered off, and the filtrate was concentrated *in vacuo*. The resulting syrupy xanthate was dissolved in benzene (15 ml) and, after the addition of morpholine (1.7 g, 19.5 mmol), the mixture was refluxed for 2 h. The mixture was then evaporated *in vacuo* to give α-mercapto-*n*-butyramide as a colorless solid, which was used in the next reaction without further purification.

The α-mercapto-*n*-butyramide thus obtained was treated with triethylamine (0.94 g, 9.4 mmol) and monochloroacetone (0.93 g, 10.0 mmol) and worked-up in the same manner as described for **12a** to give **12b** (1.21 g, 77%) as a colorless powder, mp 69—71 °C. NMR ($CDCl_3$) δ: 1.04 (3H, d, J = 8 Hz), 1.65—1.90 (2H, m), 1.93 (3H, s), 3.12 (1H, dd, J = 6, 9 Hz), 5.14 (1H, s), 7.92 (1H, s). IR ν_{max}^{KBr} cm^{-1}: 3200, 3100, 2950, 1680. *Anal.* Calcd for $C_7H_{11}NOS$: C, 53.47; H, 7.05; N, 8.90. Found: C, 53.51; H, 7.28; N, 8.86.

5-Methyl-6-(1-ethoxycarbonyl-1,4-dihydro-4-pyridyl)-1,4-thiazin-3(4*H*)-one (9a) and the (1-Butoxycarbonyl) Analog (9b)——Ethyl chloroformate (4.78 ml, 50 mmol) was added dropwise to a stirred solution of pyridine (4.85 ml, 60 mmol) in acetonitrile (21 ml) at 0 °C and the mixture was stirred at the same temperature for 10 min. Compound **4** (0.65 g, 5 mmol) was then added portionwise, and the reaction mixture was stirred at 0 °C for 20 min and then at room temperature for 5 h. The mixture was evaporated to dryness *in vacuo* and the residue was dissolved in $CHCl_3$, washed with 2 N HCl solution and then with water, and dried over anhydrous $MgSO_4$. The product obtained by removal of the solvent *in vacuo* was chromatographed on silica gel (ethyl acetate–hexane = 2 : 1) then recrystallized from EtOH to give **9a** (0.39 g, 27.7%) as pale yellow needles, mp 145.5—146 °C. NMR ($CDCl_3$) δ: 1.33 (3H, t, J = 7 Hz), 2.00 (2H, s), 3.25 (2H, s), 4.21 (1H, d, J = 3 Hz), 4.32 (2H, q, J = 7 Hz), 4.75 (2H, dd, J = 3, 8 Hz), 7.02 (2H, d, J = 8 Hz), 8.90 (1H, s). IR ν_{max}^{KBr} cm^{-1}: 3200, 3090, 2990, 2920, 1725, 1680, 1640. *Anal.* Calcd for $C_{13}H_{16}N_2O_3S$: C, 55.69; H, 5.75; N, 9.99. Found: C, 55.44; H, 5.80; N, 9.74.

By using di-*tert*-butyl dicarbonate instead of ethyl chloroformate, the 1-butoxycarbonyl analog (**9b**) was synthesized in the same manner in 17% yield as pale yellow needles, mp 155—158 °C.

5-Methyl-6-(4-pyridyl)-2*H*-1,4-thiazin-3(4*H*)-one (6a) and Its Analogs (6b—e and 14a—b)——A mixture of **5a** (300 g, 782.3 mmol), sublimed sulfur (150 g, 4.69 mmol) and dimethylformamide (DMF) (1.5 l) was refluxed for 1.5 h. The mixture was evaporated to dryness *in vacuo*, and the residue was extracted with 2 N HCl solution. The insoluble material was filtered off and the filtrate was washed with $CHCl_3$ and neutralized with 2 N NaOH solution below 0 °C. The resulting precipitates were collected and washed with water. Recrystallization from DMF–H_2O gave **6a** (124 g, 77%) as pale yellow prisms.

Concentrated HCl (14 ml) was added dropwise to a suspension of **6a** (20.5 g, 99.5 mmol) in MeOH–H_2O (500 ml, 3 : 2, v/v) and the mixture was stirred at room temperature for 0.5 h. The solvent was evaporated off *in vacuo* and the residue was washed with acetone (200 ml) and recrystallized from EtOH to give the hydrochloride of **6a** (21 g, 87%) as yellow needles, mp >250 °C (dec.). *Anal.* Calcd for $C_{10}H_{10}N_2OS \cdot HCl$: C, 49.48; H, 4.56; N, 11.54. Found: C, 49.44; H, 4.66; N, 11.64.

The sulfate [yellow needles from EtOH, mp 242—243 °C (dec.)] and the tosylate [yellow needles from EtOH, mp 205—206 °C (dec.)] were analogously obtained. Compounds **6b—e** and **14a—b** were also obtained in the above manner. The yields, melting points, spectral data and elemental analyses are given in Table III.

5-Methyl-6-(3-methoxycarbonyl-4-pyridyl)-2*H*-1,4-thiazin-3(4*H*)-one (6f) and Its 3-Acetylpyridyl Analog (6g)——A suspension of **5f** (2.1 g, 4.8 mmol) and zinc powder (1.5 g, 22.9 mmol) in THF–H_2O (60 ml, 1 : 1, v/v) was stirred at 60 °C for 2 h. After addition of zinc powder (0.5 g, 7.6 mmol), the mixture was further stirred at the same temperature for 2 h, then allowed to cool. The insoluble material was filtered off and washed with THF–H_2O (1 : 1, v/v). The combined filtrate and washings were extracted with benzene. The extract was washed with water, dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo* to give **10f** (0.76 g, 60% as a pale yellow powder, mp 176—177 °C which was submitted to the next reaction without further purification. NMR (DMSO-d_6) δ: 1.91 (3H, s), 3.05 (2H, ABq, J = 14 Hz), 3.54 (3H, s), 4.39 (1H, d, J = 5 Hz), 4.45 (1H, dd, J = 5, 8 Hz), 6.14 (1H, dd, J = 6, 8 Hz), 7.24 (1H, d, J = 6 Hz), 8.30 (1H, br), 9.34 (1H, s). IR ν_{max}^{KBr} cm^{-1}: 3200, 1620, 1600.

In an analogous manner, **10g**, was synthesized in 55% yield as pale yellow needles, mp 203—205 °C (dec.). NMR (DMSO-d_6) δ: 1.93 (3H, s), 2.05 (3H, s), 2.96 (2H,s), 4.43 (1H, d, J = 5 Hz), 4.60 (1H, dd, J = 5, 7 Hz), 6.16 (1H, dd, J = 5, 7 Hz), 7.43 (1H, d, J = 6 Hz), 8.43 (1H, m), 9.30 (1H, s).

A suspension of **10f** (0.76 g, 2.9 mmol) and DDQ (0.65 g, 2.9 mmol) in acetonitrile (30 ml) was stirred at 50 °C for 2 h, then allowed to cool. The insoluble material was filtered off and the filtrate off and the filtrate was evaporated to dryness *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH = 20 : 1) and recrystallized from

EtOH to give **6f** (0.48 g, 62.3%) as pale yellow needles, mp 177—178 °C.

Compound **6g** was synthesized analogously. The yields, spectral data and elemental analyses are given in Table III.

5-Methyl-6-(4-piperidyl)-2*H*-1,4-thiazin-3(4*H*)-one (11)——Zinc powder (1.7 g, 26 mmol) was added to a stirred solution of **5a** (1 g, 2.6 mmol) in formic acid (14 ml) and the mixture was stirred at room temperature for 3 h. The insoluble materials were filtered off, and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water (30 ml) and the resulting solution was adjusted to pH 7.0 with 1 N NaOH solution and then extracted with $CHCl_3$. The extracts were worked-up in a usual manner and chromatographed on silica gel ($CHCl_3$–MeOH) to give **6a** (20 mg, 3.7%).

The aqueous solution was adjusted to pH 12 with 1 N NaOH solution and extracted three times with 50 ml portions of $CHCl_3$. The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo* to give **11** (200 mg, 36.2%) as a pale yellow powder, mp 180—195 °C (dec.). NMR ($CDCl_3$) δ: 1.65 (5H, m), 1.97 (3H, s), 2.60 (3H, m), 3.15 (4H, s+t), 8.25 (1H, s). IR ν_{max}^{KBr} cm^{-1}: 3300, 3200, 3050, 1680, 1640. *Anal.* Calcd for $C_{10}H_{16}N_2OS$: C, 57.12; H, 4.79; N, 13.32. Found: C, 57.05; H, 4.40; N, 13.10.

5-Methyl-6-(4-pyridazinyl)-2*H*-1,4-thiazin-3(4*H*)-one (16a) and Its 3-Methylpyridazinyl Analog (16b)——A mixture of pyridazine (1.44 g, 19.8 mmol), 2,2,2-trichloroethyl chloroformate (3.44 ml, 25.0 mmol), **4** (1.29 g, 10.5 mmol) and acetonitrile (40 ml) was treated in the same manner as described for **5a**. After work-up, the crude product was chromatographed on silica gel (ethyl acetate–hexane = 1 : 1) and recrystallized from MeOH to give **15a** (2.4 g, 63%) as colorless needles. Compound **15b** was analogously obtained from 3-methylpyridazine.

A mixture of 15a (3.11 g, 8.1 mmol), sublimed sulfur (1.5 g, 46.9 mmol) and DMF (15 ml) was treated in the same manner as described for **6a**. Recrystallization from MeOH gave **16a** (1.02 g, 23%) as pale yellow plates.

Compound **16b** was also analogously obtained. The yields, melting points, spectral data and elemental analyses are given in Tables II and III.

5-Methyl-6-(4-quinolyl)-2*H*-1,4-thiazin-3(4*H*)-one (19a) and Its 6-Methoxyquinolyl Derivative (19b)——A mixture of quinoline (10.0 g, 78.0 mmol), 2,2,2-trichloroethyl chloroformate (16.4 g, 78.0 mmol), **4** (5 g, 39.0 mmol) and acetonitrile (250 ml) were treated in the same manner as described for **5a**. The crude product was chromatographed on silica gel to give **17a** (4.0 g, 24%) as pale yellow plates, mp 168—170 °C (from EtOH) and then **18a** (1.0 g, 6%) as pale yellow plates, mp 199.5—200 °C (from EtOH). The *Rf* values on TLC (ethyl acetate–hexane = 2 : 3) were 0.38 for **17a** and 0.48 for **18a**. Compounds **17b** and **18b** were obtained in the same manner.

A mixture of **17a** (4.0 g, 9.2 mmol), sublimed sulfur (2.5 g, 78.1 mmol) and DMF (15 ml) was refluxed for 3.5 h and then treated in the same manner as described for **6a**. The crude product was chromatographed on silica gel ($CHCl_3$–MeOH = 20 : 1) and recrystallized from MeOH to give **19a** (600 mg, 25%) as colorless leaflets.

Compound **19b** was also obtained in the same manner. The yields, melting points, spectral data and elemental analyses are given in Tables II and III.

5-Methyl-6-(2-thiazolyl)-2*H*-1,4-thiazin-3(4*H*)-one (21)——Ethyl chloroformate (3.6 g, 33.2 mmol) was added dropwise to a stirred solution of thiazole (5.7 g, 67.0 mmol) in CH_2Cl_2 (72 ml) under ice-cooling and the mixture was stirred at 0 °C for 0.5 h. Compound **4** (3.6 g, 27.9 mmol) was added, and the mixture was stirred at room temperature for 5 h. The solution was washed with 2 N HCl solution and then with water, dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was chromatographed on silica gel (ethyl acetate–hexane = 1 : 1), then recrystallized from EtOH to give **20a** (1.9 g, 24%) as colorless needles.

By using 2,2,2-trichloroethyl chloroformate instead of ethyl chloroformate, **20b** was analogously obtained.

DDQ (159 mg, 0.7 mmol) was added portionwise to a stirred solution of **20a** (200 mg, 0.7 mmol) in CH_2Cl_2 (2 ml) and the mixture was stirred at room temperature for 1.5 h. The resulting precipitates were collected and washed with saturated K_2CO_3 solution and then with water. Recrystallization from MeOH gave **21** (80 mg, 54%) as colorless needles. The melting points, spectral data and elemental analyses are given in Tables II and III.

Biological Method——We determined inotropic activity using male Hartley guinea pig left atrium suspended in Krebs–Henseleit solution (gassed with 95% O_2/5% CO_2) at 30 °C. Resting tension was adjusted to 500 mg after 1 h of equilibration. The atrium was stimulated by square pulses of 5 ms duration at a voltage of 20% above threshold and a stimulating rate of 0.5 Hz. Test drug was added to the bathing fluid at 30 min intervals. Fifteen minutes after the maximum effect had been achieved, the atrium was washed with three changes of drug-free medium until the basal developed tension of the atrium was recorded isometrically on a rectilinear recorder *via* a force displacement transducer (Nihon Koden, TB-611T, Tokyo, Japan). Change of contractile force was measured as an absolute change in developed tension.

The test materials were dissolved in 0.2 N HCl to provide 3×10^{-2} M solutions, 0.1 ml aliquots of which were added to 30 ml of the bathing fluid.

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New Toxic Metabolites from a Mushroom, *Hebeloma vinosophyllum*. II.[1] Isolation and Structures of Hebevinosides VI, VII, VIII, IX, X, and XI

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Four new triterpene glycosides, hebevinosides VI, VII, VIII, and IX, whose common aglycone is 3β,7β,16β-trihydroxycucurbita-5,24-diene (hydroxyhebevinogenin), have been isolated from the acetone extract of a poisonous mushroom, *Hebeloma vinosophyllum*, and deduced to have the structures of 3β,7β,16β-trihydroxycucurbita-5,24-diene-3-*O*-β-D-xylopyranoside-16-*O*-β-D-glucopyranoside, 3β,7β,16β-trihydroxycucurbita-5,24-diene-3-*O*-β-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-β-D-glucopyranoside, 3β,7β,16β-trihydroxycucurbita-5,24-diene-3-*O*-(4-*O*-acetyl)-β-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-β-D-glucopyranoside, and 3β,7β,16β-trihydroxycucurbita-3-*O*-β-D-xylopyranoside, respectively. Two new glycosides, hebevinosides X and XI, whose common aglycone is 3β,16β-dihydroxy-7β-methoxycucurbita-5,24-diene (methoxyhebevinogenin), have also been isolated from the aqueous methanolic extract of the mushroom in addition to hebevinosides I, II, III, IV, and V previously reported, and deduced to be 3β,16β-dihydroxy-7β-methoxycucurbita-5,24-diene-3-*O*-β-D-xylopyranoside-16-*O*-β-D-glucopyranoside and 3β,16β-dihydroxy-7β-methoxycucurbita-5,24-diene-3-*O*-β-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-β-D-glucopyranoside, respectively.

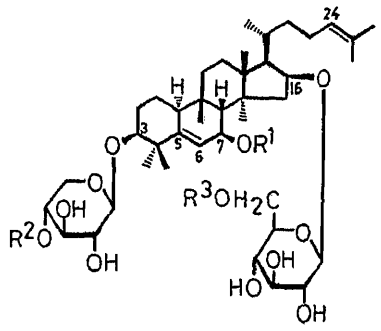
Among these eleven hebevinosides, I, IV, V, X, and XI, whose common aglycone is methoxyhebevinogenin, have been proved to be artifacts formed from the genuine metabolites III, IX, II, VI, and VII, whose common aglycone is hydroxyhebevinogenin, during extraction of the mushroom with aqueous methanol, respectively, and VIII, whose aglycone is hydroxyhebevinogenin, has also been proved to be a genuine metabolite of this mushroom.

The relationship between the structure and toxicity of hebevinosides was also investigated.

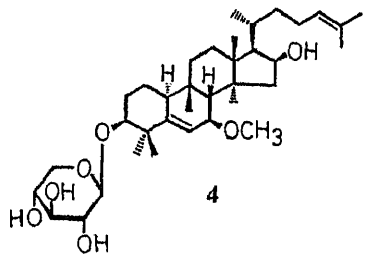
Keywords——Basidiomycetes; *Hebeloma vinosophyllum*; neurotoxin; mushroom toxin; hebevinoside; triterpene glycoside; cucurbitane; structure–toxicity relationship

Recently we reported the isolation[2] and structures of five new glycosidic metabolites, hebevinosides I (**1**), II(**2**), III(**3**), IV(**4**), and V(**5**), from the aqueous methanolic extract of a poisonous mushroom, *Hebeloma vinosophyllum* HONGO (Japanese name: akahida-wakafusatake), and demonstrated that hebevinosides I, II, III, and V were the toxic principles of this mushroom.[1] Among these hebevinosides, **1**, **4**, and **5** have methoxyhebevinogenin (3β,16β-dihydroxy-7β-methoxycucurbita-5,24-diene) (**7**) as their common aglycone, while **2** and **3** have hydroxyhebevinogenin (3β,7β,16β-trihydroxycucurbita-5,24-diene) (**6**) as their common aglycone.[1] We wish to report here the further isolation and structures of hebevinosides VI, VII, VIII, IX, X, and XI, the discrimination of genuine metabolites from artifacts among these eleven hebevinosides, and the determination of the absolute configurations of the component sugars of hebevinosides. The relationship between structure and toxicity of hebevinosides is also discussed.

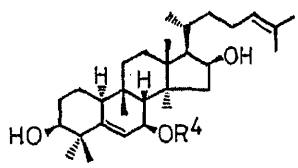
The dried fruit-bodies of *H. vinosophyllum* defatted with *n*-hexane were extracted with acetone at room temperature. During the extraction, the pH of the extracting solution was



1: $R^1=CH_3$, $R^2=H$, $R^3=COCH_3$
2: $R^1=H$, $R^2=R^3=COCH_3$



4



6: $R^4=H$
7: $R^4=CH_3$

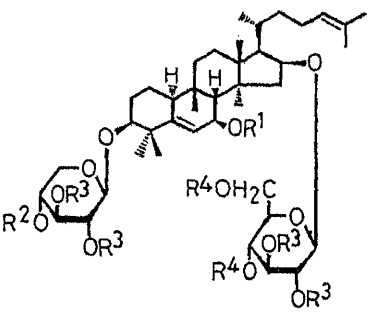
3: $R^1=R^2=H$, $R^3=COCH_3$
5: $R^1=CH_3$, $R^2=R^3=COCH_3$

Chart 1

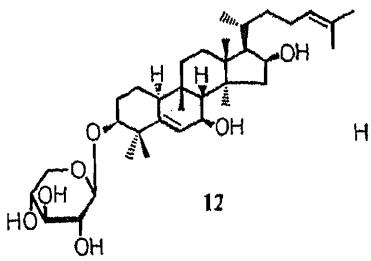
kept at seven by occasional addition of a small amount of pyridine. Successive fractionations of the acetone extract by centrifugal thin layer chromatography (TLC) using chloroform–acetone, chloroform–acetone–tetrahydrofuran (THF), and then THF as eluents and by chromatography on a Sephadex LH-20 column with chloroform, chloroform–THF, chloroform–acetone–THF, and THF as eluents afforded six glycosides, hebevinosides VIII, II, VII, IX, III, and VI (yields from the dried fruit-bodies: 0.03, 0.10, 0.04, 0.002, 0.10, and 0.09%, respectively). Among these six hebevinosides, VIII, VII, IX, and VI are new metabolites isolated from this mushroom (II and III were isolated previously[1,2]).

Comparison of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of hebevinoside VI (**8**) with that of **3**[1] indicated that **8** has no acetyl group and all the signals of **8** are quite similar to the corresponding signals of **3** except that the signals of C-4, -5, and -6 in the glucosyl moiety are shifted to 72.1 (+0.2), 78.1 (+2.5), and 63.2 (−1.6) ppm (see Table I). A consideration of the acetylation shift rule[3] suggests that **8** may be the deacetyl derivative of **3**. Actually, **8** was found to be identical with the deacetyl derivative of **3**, which was obtained from **3** by treatment with methanolic 0.1% potassium hydroxide. Thus, the structure of **8** was deduced to be 6-3-*O*-*β*-D-xylopyranoside-16-*O*-*β*-D-glucopyranoside, as shown in Chart 2.

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of hebevinoside VII (**9**) indicated that **9** has two acetyl groups (2.03 and 2.09 ppm (each 3H, s, $-COCH_3$)). On acetylation, **9** afforded a hexaacetate which was identical with the heptaacetate of **3** (**10**).[1] Comparison of the ^{13}C-NMR spectrum of **9** with that of **8** indicated that all signals are quite



8: $R^1=R^2=R^3=R^4=H$
9: $R^1=R^2=R^3=H$, $R^4=COCH_3$
10: $R^1=R^2=R^3=R^4=COCH_3$
11: $R^1=R^3=H$, $R^2=R^4=COCH_3$



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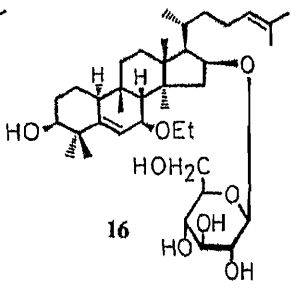

16

13: $R^1=CH_3$, $R^2=R^4=COCH_3$, $R^3=H$
14: $R^1=CH_3$, $R^2=R^3=R^4=H$
15: $R^1=CH_3$, $R^2=R^3=H$, $R^4=COCH_3$

Chart 2

TABLE I. ^{13}C-NMR Data for Hebevinosides III (**3**), VI (**8**), VII (**9**), VIII (**11**), X (**14**), XI (**15**), and the Derivative **13**, δ (ppm) from TMS in C_5D_5N

Position	3[1]	8	9	11	13	14	15
3	87.4	87.4	87.4	87.4	87.4	87.4	87.5
5	146.3	146.2	146.4	146.3	147.9	147.7	148.0
6	122.4	122.6	122.5	122.6	119.5	119.4	119.4
7	67.5	67.3	67.4	67.5	77.8	77.6	77.8
16	82.2	81.9	82.5	82.5	82.5	82.1	82.5
24	126.8	126.8	126.9	126.9	126.9	126.8	126.9
25	129.9	130.0	130.1	130.1	130.1	130.0	130.1
OMe					56.3	56.2	56.3
Xylose-1	107.7	107.6	107.6	107.4	107.6	107.7	107.8
Xylose-2	75.1	75.0	75.1	75.2	75.1	74.9	74.9
Xylose-3	78.4	78.5	78.5	74.9	74.9	78.5	78.5
Xylose-4	71.1	71.1	71.1	73.2	73.1	71.1	71.1
Xylose-5	66.8	67.1	67.1	63.2	63.2	67.0	67.1
Glucose-1	106.6	106.6	106.5	106.5	106.7	106.8	106.7
Glucose-2	75.0	75.7	75.6[a]	75.6[b]	75.5	75.6	75.5
Glucose-3	78.6	78.6	75.5[a]	75.5[b]	75.5	78.7	75.5
Glucose-4	71.9	72.1	72.4	72.4	72.4[c]	72.0	72.3[d]
Glucose-5	75.6	78.1	72.4	72.4	72.3[c]	78.2	72.4[d]
Glucose-6	64.8	63.2	63.6	63.6	63.5	63.2	63.5
$CO\underline{C}H_3$	20.8		20.8	20.8	20.8		20.8
			20.9	20.9	20.9		20.9
				20.9	20.9		
$\underline{C}OCH_3$	170.8		170.5	170.5	170.3		170.3
			170.7	170.6	170.6		170.5
				170.7	170.6		

a—d) Assignments may be reversed.

similar to the corresponding signals of **8**, except that the signals of C-3, -4, -5, and -6 in the glucosyl moiety are shifted to 75.5 (−3.1), 72.4 (+0.3), 72.4 (−5.7), and 63.6 (+0.4) ppm (see Table I), respectively. The additivity rule of acetylation shift parameters[4] indicates that **9** may be a diacetylated derivative of **8** and the two acetyl groups in **9** should be present at C-4 and -6 in the glucosyl moiety. Thus, the structure of **9** was deduced to be 6-3-*O*-*β*-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-*β*-D-glucopyranoside. The signals of hydrogens attached to the carbons bearing acetoxyl groups in **9** were partly overlapped with other signals in the ^{1}H-NMR spectrum, so that they were not completely assigned.

The ^{1}H-NMR spectrum of hebevinoside VIII (**11**) showed that **11** has three acetyl groups (2.10, 2.12, and 2.12 ppm (each 3H, s, $-COCH_3$)). On acetylation, **11** afforded a pentaacetate which was identical with **10**.[1] Comparison of the ^{13}C-NMR spectrum of **11** with that of **9** indicated that all signals are quite similar to the corresponding signals of **9**, except that the signals of C-3, -4, and -5 in the xylosyl moiety are shifted to 74.9 (−3.6), 73.2 (+2.1), and 63.2 (−3.9) ppm, respectively (see Table I). The results suggested that **11** has three acetyl groups: one would be present at C-4 in the xylosyl moiety, and the other two at C-4 and -6 in the glucosyl moiety, as in **9**. Furthermore, the signals of four hydrogens attached to carbon bearing an acetoxyl group are observed at 4.07 (1H, dd, J_1=12.1, J_2=4.8 Hz), 4.16 (1H, dd, partly overlapped with other signals), 4.81 (1H, dt, $J_1=J_2$=8.1, J_3=5.1 Hz), and 4.91 ppm (1H, t, J=9.5 Hz) in the ^{1}H-NMR spectrum of **11**. The signal at 4.81 ppm was assigned to H-4 in the xylosyl moiety, and those at 4.07, 4.16, and 4.91 ppm to H-6a, -6b, and -4 in the glucosyl moiety in **11**, respectively, on the basis of spin decoupling measurements and with reference to the ^{1}H-NMR spectral data of acetylated derivatives of xylose and glucose in the

literature.[5] Thus, the structure of **11** was deduced to be **6**-3-*O*-(4-*O*-acetyl)-*β*-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-*β*-D-glucopyranoside.

The ^{1}H-NMR spectrum of hebevinoside IX (**12**) was quite similar to that of **4**[1] except that **12** has a hydroxyl group instead of a methoxyl group. Stirring of a solution of **12** in 90% methanol at pH 4 at room temperature for 5 h afforded a product which was identical with **4** in terms of ^{1}H-NMR spectrum and TLC. Comparison of the ^{1}H-NMR spectrum of **12** (3.89(1H, d, J=4.9 Hz, H-7), and 5.68 ppm (1H, d, J=4.9 Hz, H-6)) with that of **4** (3.34(3H, s, OCH_3), 3.42 (1H, d, J=5.6 Hz, H-7) and 5.74 ppm (1H, d, J=5.6 Hz, H-6))[1] suggested that the replacement of a hydroxyl group at position 7*β* in **12** by a methoxyl had occurred to give **4** during the treatment of **12** in 90% methanol at pH 4. Accordingly, the structure of **12** was deduced to be **6**-3-*O*-*β*-D-xylopyranoside.

Application of the same treatment to **11** afforded a product (**13**). Comparison of the ^{1}H-NMR spectrum of **13** (3.33(3H, s, OCH_3), 3.39 (1H, d, J=4.9 Hz, H-7), and 5.74 (1H, d, J=4.9 Hz, H-6)) with that of **11** (3.93(1H, d, J=5.5 Hz, H-7) and 5.74 ppm (1H, d, J=5.5 Hz, H-6)) indicated that the replacement of the hydroxyl group at position 7*β* in **11** by a methoxyl had occurred to give **13** (7-3-*O*-(4-*O*-acetyl)-*β*-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-*β*-D-glucopyranoside) during the treatment of **11** as well as that of **12** in 90% methanol at pH 4. The treatment of **12** or **11** with 90% methanol at pH 4 is considered to be a model reaction of the extraction of the mushroom with 90% methanol, because the pH of the extracting solution was found to be about 4 during extraction. These considerations indicated that **1**, **4**, and **5**, which have **7** as their common aglycone, were artificially formed from their genuine glycosides **3**, **12**, and **2**, respectively, having **6** as their common aglycone, during extraction of the mushroom with 90% methanol.

Recently, we have further isolated two artificial glycosides named hebevinosides X (**14**) and XI (**15**) along with **1**—**5** previously reported[1] from the aqueous methanolic extract of the mushroom.

The ^{1}H-NMR spectrum of **14** showed that **14** has no acetyl group. Comparison of the ^{13}C-NMR spectrum of **14** with that of **1**[1] indicated that all signals of **14** are quite similar to the corresponding signals of **1** except that the signals of C-4, -5, and -6 in the glucosyl moiety are shifted to 72.0 (+0.3), 78.2 (+2.7), and 63.2 (−1.5) ppm (see Table I). This indicated on the basis of the acetylation shift rule[3] that **14** may be a deacetyl derivative of **1**. Actually, **14** was identical with the deacetyl derivative of **1** obtained on alkaline treatment of **1**.[1] Thus, the structure of **14** was deduced to be **7**-3-*O*-*β*-D-xylopyranoside-16-*O*-*β*-D-glucopyranoside.

Comparison of the ^{1}H-NMR spectrum of **15** with that of **9** suggested that **15** may be the 7*β*-methoxylated derivative of **9**, because all signals of **15** are quite similar to the corresponding signals of **9** except that the signals of OCH_3-7*β*, H-7*α*, and H-6 are observed at 3.33 (3H, s), 3.38 (1H, d, J=6.0 Hz), and 5.73 (1H, d, J=6.0 Hz), respectively, in the ^{1}H-NMR spectrum of **15**. Indeed, **15** was identical with the 7*β*-methoxylated derivative of **9**, which was obtained on treatment of **9** with 90% methanol at pH 4. Thus, the structure of **15** was deduced to be **7**-3-*O*-*β*-D-xylopyranoside-16-*O*-(4,6-di-*O*-acetyl)-*β*-D-glucopyranoside. This conclusion was also supported by the ^{13}C-NMR spectral data (see Table I).

The ^{1}H-NMR data for H-6 and -7 in hebevinosides indicated that the configuration of the methoxyl group at position 7 in all artificial hebevinosides, **1**, **4**, **5**, **14**, and **15**, is *β*, like that of the hydroxyl group at position 7 in all the genuine hebevinosides, **2**, **3**, **8**, **9**, **11**, and **12**.

Previously, we suggested that both xylose and glucose of hebevinosides probably belong to the D-series, because most sugars isolated as fungal metabolites belong to the D-series.[1]

Methyl 2,3,4-tri-*O*-methyl-*β*-xyloside and methyl 2,3,4,6-tetra-*O*-methyl-*α*-glucoside obtained from a mixture of **1** and **2** by methanolysis with methanolic 2 N hydrochloric acid followed by Hakomori's methylation[6] were identical with authentic methyl 2,3,4-tri-*O*-methyl-*β*-D-xylopyranoside and methyl 2,3,4,6-tetra-*O*-methyl-*α*-D-glucopyranoside, respec-

tively, in terms of optical rotatory dispersion (ORD) curves and behavior on gas liquid chromatography (GLC). Accordingly, both xylose and glucose of hebevinosides were proved to belong to the D-series.

On intraperitoneal administration of 100 mg/kg of **2** or **3** to mice, four among five mice died after paralysis. On the other hand, on administration of 50 mg/kg of **5** and **1**, three among four and all of five mice died after paralysis, respectively. Therefore, substitution of a hydroxyl group with a methoxyl group at position 7β in hebevinosides increases the toxicity. On administration of 100 mg/kg of **8**, all of four mice died after paralysis, but, on administration of 50 mg/kg of **8**, all of five mice survived. The LD_{50} value of **8** on intraperitoneal administration to mice was found to be 66 mg/kg by the up-and-down method.[7] On administration of 200 mg/kg of 4, which has a xylosyl moiety at position 3, a methoxyl group at position 7, and a hydroxyl group at position 16, all of five mice survived. On the other hand, on administration of 100 mg/kg of a partially hydrolyzed compound derived from the deacetyl derivative of 1 by enzymatic hydrolysis in aqueous ethanol, 16,[1] which has a hydroxyl group at position 3, an ethoxyl group at position 7, and a glucosyl moiety at position 16, all of three mice died after paralysis. This result suggests that the presence of a glucosyl moiety at position 16 in hebevinosides is indispensable for the appearance of the toxicity.

Experimental

All melting points were measured on a Yanagimoto micro-melting point apparatus (hot stage type) and are uncorrected. The optical rotations were measured with a JASCO DIP-181 digital polarimeter. The ultraviolet (UV) spectra were recorded with a Hitachi 323 recording spectrophotometer, the infrared (IR) spectra with a Hitachi EPI-G3 grating infrared spectrophotometer, the electron impact mass spectra (EI-MS) with a Hitachi M-60 or a Hitachi RMU-7M mass spectrometer, the ORD spectra with a JASCO J-20 recording spectropolarimeter, the ^{1}H-NMR spectra with a JEOL JNM-GX270 FT-NMR spectrometer at 270 MHz, and the ^{13}C-NMR spectra with a JEOL JNM-GX270 FT-NMR spectrometer at 67.8 MHz. Chemical shifts are expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard. The following abbreviations are used; s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; dt, doublet of triplet; m, multiplet; br, broad. The TLC analyses were carried out with silica gel plates (Merck Kieselgel 60 G) and the GLC analyses with a column of 5% neopentylglycol succinate polyester on Chromosorb W-HP (80—100 mesh; 3 mm × 2.0 m; column temp., 155 °C; carrier gas, N_2 40 ml/min) on a Shimadzu GC-5A gas chromatograph equipped with a hydrogen flame ionization detector. Column chromatographic separations were carried out with columns of silica gel (Mallinckrodt Silic AR CC-7 or Kusano C.I.G. column system) or octadecyl silica gel (Hitachi 3056) or Sephadex LH-20 (Pharmacia). Centrifugal thin layer chromatographic separations were carried out with disks of silica gel (Merck Kieselgel 60 G including $CaSO_4 \cdot 1/2H_2O$ (3.5% $CaSO_4 \cdot 1/2H_2O$ in Kieselgel 60 G)) on a Harrison chromatotron. The toxicity of each sample was examined by intraperitoneal injection of 0.1 ml of a solution of each sample in dimethyl sulfoxide (DMSO) into each mouse (ddY, male, 20—25 g) and by observation of the behavior of the injected mice within 72 h (3—5 mice were employed for each sample).

Isolation of Hebevinosides VI (8), VII (9), VIII (11), and IX (12)——Dried fruit-bodies (150.0 g) obtained by cultivation of *H. vinosophyllum*[1] were cut into fine pieces and shaken in *n*-hexane (1.0 l) at room temperature for a few hours five times to remove fatty components (0.74 g). The defatted fruit-bodies were extracted with acetone (1.0 l), whose pH was kept at 7 by occasional addition of a small amount of pyridine, at room temperature for 5 h seven times to afford the acetone extract (4.95 g). The acetone extract was separated by centrifugal TLC with $CHCl_3$–acetone (1 : 1, v/v), $CHCl_3$–acetone–THF (1 : 1 : 0.1, v/v), (1 : 1 : 0.2, v/v), (1 : 1 : 0.5, v/v), and THF only. A mixture of the latter half of the fraction eluted with $CHCl_3$–acetone (1 : 1) and the first half of the fraction eluted with $CHCl_3$–acetone–THF (1 : 1 : 0.1) (710 mg) was further chromatographed on a Sephadex LH-20 (23 g) column with $CHCl_3$ and $CHCl_3$–THF (20 : 1, v/v) to afford **11** (45 mg). A mixture (720 mg) of the latter half of the fraction eluted with $CHCl_3$–acetone–THF (1 : 1 : 0.1) and the fraction eluted with $CHCl_3$–acetone–THF (1 : 1 : 0.2) was further chromatographed on a Sephadex LH-20 (48 g) column with $CHCl_3$–THF (10 : 1, v/v) to afford **2** (150 mg), **9** (60 mg), and **12** (3 mg). A mixture (1.25 g) of the fraction eluted with $CHCl_3$–acetone–THF (1 : 1 : 0.5) and the first half of the fraction eluted with THF was further chromatographed on a Sephadex LH-20 (90 g) column with $CHCl_3$–THF (10 : 1) and THF to afford **3** (150 mg) and **8** (135 mg).

Hebevinoside VI (**8**): Amorphous. $[\alpha]_D^{23} + 56°$ (c = 0.054, pyridine). UV in CH_3CN: end absorption. IR ν_{max}^{KBr} cm^{-1}: 3380, 1635, 1075, 1035. Identical with the deacetyl derivative of **3** in terms of ^{1}H-NMR spectra (C_5D_5N), ^{13}C-NMR

spectra (C_5D_5N), IR spectra (KBr), and TLC behavior (solvent: $CHCl_3$–MeOH (4:1, v/v)).

Hebevinoside VII (**9**): Amorphous. $[\alpha]_D^{20}+82°$ ($c=0.22$, acetone). EI-MS *m/z* (%): 818 (9.0, M^+-H_2O), 686 (18.5, $M^+-C_5H_{10}O_5$(xyl.)), 668 (10.2, $M^+-H_2O-C_5H_{10}O_5$), 653 (21.3, $M^+-H_2O-C_5H_{10}O_5-CH_3$). UV in CH_3CN: end absorption. IR $\nu_{max}^{KBr}cm^{-1}$: 3450, 1735, 1630, 1070, 1035. ^{1}H-NMR (C_5D_5N): 0.86, 1.08, 1.19, 1.38, 1.63 (each, 3H, s), 1.03 (3H, d, $J=6.0$ Hz, H_3-21), 1.71, 1.75 (each 3H, s, H_3-26 and H_3-27), 2.03, 2.09 (each 3H, s, Ac), 4.43 (1H, partly overlapped with other signals, H-7), 4.66 (1H, d, $J=7.7$ Hz, H-glc.1), 4.82 (1H, d, $J=7.7$ Hz, H-xyl.1), 5.51 (1H, t, $J=9.8$ Hz, H-24), 6.12 (1H, d, $J=4.9$ Hz, H-6). **9** (1 mg) was acetylated with Ac_2O (1 drop) in pyridine (2 drops) at room temperature overnight to afford a hexaacetate (1 mg), amorphous, which was identical with the heptaacetate of **3** (**10**)[1] in terms of ^{1}H-NMR ($CDCl_3$) and TLC behavior (solvent: $CHCl_3$–MeOH (50:1, v/v)).

Hebevinoside VIII (**11**): Amorphous. $[\alpha]_D^{20}+36.5°$ ($c=0.52$, $CHCl_3$). EI-MS *m/z* (%): 860 (47.1, M^+-H_2O), 686 (27.4, $M^+-C_7H_{12}O_6$ (Ac-xyl.)), 668 (83.9, $M^+-H_2O-C_7H_{12}O_6$), 653 (36.5, $M^+-H_2O-C_7H_{12}O_6-CH_3$). UV in CH_3CN: end absorption. IR $\nu_{max}^{KBr}cm^{-1}$: 3420, 1740, 1640, 1075, 1025. ^{1}H-NMR ($CDCl_3$): 0.66, 1.00, 1.08, 1.22, 1.26 (each 3H, s), 0.95 (3H, d, $J=6.6$ Hz, H_3-21), 1.61, 1.68 (each 3H, s, H_3-26 and H_3-27), 2.10, 2.12, 2.12 (each 3H, s, Ac), 3.29 (1H, dd, $J_1=11.7$, $J_2=8.1$ Hz, H-xyl.5a), 3.55 (1H, m, H-glc.5), 3.62 (1H, t, $J=9.5$ Hz, H-glc.3), 3.70 (1H, t, $J=8.1$ Hz, H-xyl.3), 3.93 (1H, d, $J=5.5$ Hz, H-7), 3.95 (1H, dd, partly overlapped with the signal at 3.93 ppm, H-xyl.5b), 4.07 (1H, dd, $J_1=12.1$, $J_2=4.8$ Hz, H-glc.6a), 4.16 (1H, dd, partly overlapped with other signals, H-glc.6b), 4.22 (1H, d, $J=5.6$ Hz, H-glc.1), 4.40 (1H, d, $J=5.9$ Hz, H-xyl.1), 4.81 (1H, dt, $J_1=J_2=8.1$, $J_3=5.1$ Hz, H-xyl.4), 4.91 (1H, t, $J=9.5$ Hz, H-glc.4), 5.14 (1H, t, $J=6.8$ Hz, H-24), 5.74 (1H, d, $J=5.5$ Hz, H-6). **11** (2 mg) was acetylated with Ac_2O (1 drop) in pyridine (2 drops) at room temperature overnight to afford a pentaacetate (2 mg), amorphous, which was identical with **10** in terms of ^{1}H-NMR ($CDCl_3$) and TLC behavior (solvent: $CHCl_3$–MeOH (50:1, v/v)).

Hebevinoside IX (**12**): Amorphous. ^{1}H-NMR ($CDCl_3$): 0.61, 0.93, 0.97, 1.02, 1.15 (each 3H, s), 0.90 (3H, d, $J=6.2$ Hz, H_3-21), 1.54, 1.62 (each 3H, s, H_3-26 and H_3-27), 3.89 (1H, d, $J=4.9$ Hz, H-7), 4.33 (1H, d, $J=5.9$ Hz, H-xyl.1), 5.10 (1H, t, $J=6.2$ Hz, H-24), 5.68 (1H, d, $J=4.9$ Hz, H-6).

Deacetylation of 3——A solution of **3** (40 mg) in methanolic 0.1% KOH (3.0 ml) was stirred at room temperature for 30 min. The reaction mixture was neutralized with Amberlite MB-3 and evaporaed *in vacuo* to afford a product mixture (36 mg), which was chromatographed on a column of silica gel (3.0 g) with C_6H_6–acetone (1:1, v/v) to give the 7β-methoxy deacetyl derivative of **3** (4 mg), a mixture (8 mg) of the 7β-methoxy deacetyl derivative of **3** and the deacetyl derivative of **3**, and the deacetyl derivative of **3** (16 mg). The 7β-methoxy deacetyl derivative of **3** was identical with the deacetyl derivative of **1**[1] and also with **14** in terms of ^{1}H-NMR spectra (C_5D_5N), ^{13}C-NMR spectra (C_5D_5N), IR spectra (KBr), and TLC behavior (solvent: $CHCl_3$–MeOH (4:1, v/v)). The deacetyl derivative of **3**: Amorphous. ^{1}H-NMR (C_5D_5N): 0.78, 1.12, 1.17, 1.35, 1.63 (each 3H, s), 1.04 (3H, d, $J=6.4$ Hz, H_3-21), 1.68, 1.72 (each 3H, s, H_3-26 and H_3-27), 4.27 (1H, d, $J=4.8$ Hz, H-7), 4.74, 4.81 (each 1H, d, $J=7.9$ Hz, H-glc.1 and H-xyl.1), 5.52 (1H, t, $J=7.1$ Hz, H-24), 6.03 (1H, d, $J=4.8$ Hz, H-6). $IR_{max}^{KBr}cm^{-1}$: 3380, 1635, 1075, 1035.

Transformation of 12 to 4——The pH value of a solution of **12** (14 mg) in 90% MeOH (1.0 ml) was adjusted to 4 by addition of one drop of 0.1 N HCl. The solution was stirred at room temperature for 5 h. The reaction mixture was then diluted with water and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water, dried over Na_2SO_4, and evaporated *in vacuo* to afford a product mixture, which was chromatographed on a column of silica gel (Kusano C.I.G. column system, 1.8 cm diameter × 30 cm length) with $CHCl_3$–MeOH (10:1, v/v) under 38 kg/cm^2 pressure to give a crude product (5 mg). The crude product was further chromatographed on a column of Sephadex LH-20 (23 g) with $CHCl_3$ to afford a product (1.5 mg), which was identical with authentic **4** in terms of ^{1}H-NMR spectra ($CDCl_3$) and TLC behavior (solvent: $CHCl_3$–MeOH (7:1, v/v)).

Transformation of 11 to 13——The pH value of a solution of **11** (10 mg) in 90% MeOH (1.0 ml) was adjusted to 4 by addition of one drop of 0.1 N HCl. The solution was treated in the same way as for the transformation of **12** to **4** to afford a product mixture (10 mg), which was purified on a column of octadecyl silica gel (1.0 cm diameter × 25 cm length) with $CHCl_3$–MeOH–H_2O (16.5:32.5:1, v/v) under 60 kg/cm^2 pressure to give **13** (6.5 mg), amorphous. ^{1}H-NMR ($CDCl_3$): 0.68, 0.98, 1.01, 1.12, 1.26 (each 3H, s), 0.96 (3H, d, $J=6.1$ Hz, H_3-21), 1.61, 1.69 (each 3H, s, H_3-26 and H_3-27), 2.07, 2.10, 2.11 (each 3H, s, Ac), 3.33 (3H, s, OMe), 3.39 (1H, d, $J=4.9$ Hz, H-7), 4.06 (1H, dd, $J_1=11.9$, $J_2=4.6$ Hz, H-glc.6a), 4.18 (1H, dd, partly overlapped with other signals, H-glc.6b), 4.20 (1H, d, $J=7.9$ Hz, H-glc.1), 4.40 (1H, d, $J=6.1$ Hz, H-xyl.1), 4.81 (1H, dt, $J_1=J_2=7.5$, $J_3=4.2$ Hz, H-xyl.4), 4.92 (1H, t, $J=9.2$ Hz, H-glc.4), 5.14 (1H, t, $J=6.4$ Hz, H-24), 5.74 (1H, d, $J=4.9$ Hz, H-6).

Isolation of Hebevinosides X (14) and XI (15)——From the 90% MeOH extract (50.5 g) of the dried fruit-bodies (277.6 g) of cultivated *H. vinosophyllum*, 20.0 g of *n*-BuOH-soluble fraction had been obtained. Repeated column chromatography of the *n*-BuOH-soluble fraction on silica gel with C_6H_6–acetone and on Sephadex LH-20 with $CHCl_3$–MeOH had afforded **4** (56 mg), **5** (112 mg), **2** (250 mg), **1** (472 mg), and **3** (278 mg), as described in our previous reports.[1,2] A residual chromatographic fraction from which **4** and **5** had previously been separated was further chromatographed on a column of silica gel with C_6H_6–acetone (5:1, v/v) and (2:1, v/v) to afford **15** (55 mg), amorphous. $[\alpha]_D^{20}+65°$ ($c=0.17$, MeOH). UV in MeOH: end absorption. $IR_{max}^{KBr}cm^{-1}$: 3450, 1747, 1645, 1085, 1040. This product was identical with the 7β-methoxylated derivative of **9** in terms of ^{1}H-NMR spectra ($CDCl_3$) and TLC behavior (solvent: $CHCl_3$–MeOH (4:1, v/v)).

A residual chromatographic fraction from which **3** had previously been separated was further chromatographed on a column of silica gel with C_6H_6–acetone (1 : 1, v/v) to afford **14** (220 mg), amorphous. $[\alpha]_D^{20}$ +63° (c=0.49, MeOH). UV in MeOH: end absorption. IR_{max}^{KBr} cm^{-1}: 3400, 1640, 1075, 1035. ^{1}H-NMR (C_5D_5N): 0.74, 1.10, 1.16, 1.24, 1.63 (each 3H, s), 1.08 (3H, d, J=7.3 Hz, H_3-21), 1.69, 1.73 (each 3H, s, H_3-26 and H_3-27), 3.21 (3H, s, OMe), 3.40 (1H, d, J=5.3 Hz, H-7), 4.79 (2H, d, J=7.6 Hz, H-glc.1 and H-xyl.1), 5.55 (1H, t, J=6.8 Hz, H-24), 5.88 (1H, d, J=5.3 Hz, H-6).

7β-Methoxylation of 9——A solution of **9** (1 mg) in 90% MeOH (0.2 ml) was adjusted to pH 4 by addition of 0.1 N HCl, and stirred at room temperature for 2.5 h. The reaction mixture was evaporated *in vacuo* under ice-cooling to afford the 7β-methoxylated derivative of **9** (1 mg), amorphous. ^{1}H-NMR ($CDCl_3$): 0.68, 0.97, 1.01, 1.12, 1.21 (each 3H, s), 0.96 (3H, d, J=5.0 Hz, H_3-21), 1.60, 1.69 (each 3H, s, H_3-26 and H_3-27), 2.07, 2.12 (each 3H, s, Ac), 3.33 (3H, s, OMe), 3.38 (1H, d, J=6.0 Hz, H-7), 4.00 (1H, dd, J_1=12.0, J_2=4.8 Hz, H-glc.6a), 4.16 (1H, dd, partly overlapped with other signals, H-glc.6b), 4.19 (1H, d, J=7.2 Hz, H-glc.1), 4.38 (1H, d, J=6.0 Hz, H-xyl.1). 4.92 (1H, t, J=8.8 Hz, H-glc.4), 5.14 (1H, brt, J=7.2 Hz, H-24), 5.73 (1H, d, J=6.0 Hz, H-6).

Methyl 2,3,4-Tri-*O*-methyl-β-D-xylopyranoside and Methyl 2,3,4,6-Tetra-*O*-methyl-α-D-glucopyranoside from a Mixture of 1 and 2——A solution of a mixture (460 mg) of **1** (253 mg) and **2** (207 mg) in methanolic 2 N HCl (2.0 ml) was refluxed for 2 h under an N_2 gas flow. The reaction mixture was diluted with water and extracted with AcOEt. The aqueous layer was neutralized with Amberlite MB-3 and lyophilized to give a mixture of methyl glycosides (160 mg), which was further methylated with NaH (about 50% in Bayol 85) (600 mg) and CH_3I (2.0 ml) in DMSO (5.0 ml) at room temperature overnight under an N_2 gas flow[6] to afford a permethylated product mixture. The product mixture was separated by centrifugal thin layer chromatography with C_6H_6–acetone (8 : 1, v/v). The first part of the chromatographic fractions was further purified by centrifugal thin layer chromatography with C_6H_6–acetone (16 : 1, v/v) to afford a resinous compound (3 mg), which was identical with authentic methyl 2,3,4-tri-*O*-methyl-β-D-xylopyranoside derived from D-xylose[6,8] in terms of ORD curve (c=0.15, $CHCl_3$) $[\alpha]^{23.5}$ (nm): −133° (589), −160° (500), −287° (400), −534° (300) ((−)-plain curve) and GLC t_R: 4.1 min. The intermediate part of the chromatographic fractions eluted with C_6H_6–acetone (8 : 1) afforded a mixture of methyl 2,3,4-tri-*O*-methyl α-D-xylopyranoside and methyl 2,3,4,6-tetra-*O*-methyl β-D-glucopyranoside (18 mg). The latter part of the chromatographic fractions eluted with C_6H_6–acetone (8 : 1) was further purified by centrifugal TLC with C_6H_6–acetone (6 : 1, v/v) to afford a resinous compound (22 mg), which was identical with authentic methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside derived from D-glucose[6,8] in terms of ORD curve (c=0.53, $CHCl_3$) $[\alpha]^{23}$ (nm): +88° (589), +120° (500), +178° (400), +399° (300), +779° (250) ((+)-plain curve) and GLC t_R: 13.1 min.

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Syntheses and Sleeping-Time-Prolonging Effect of Nitramarine and Related Compounds

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Nitramarine (**1**), which possesses a β-carboline nucleus, was synthesized by two routes. First, we applied an intramolecular thermal cyclization of the 1-azahexatriene system in heteroaromatics such as **2**. Although no intermediate (**9** or **10**) could be isolated, heating of **7** in toluene or of **8** in *o*-dichlorobenzene in the presence of hydroxylamine gave nitramarine (**1**) or its derivative (**11**), respectively. On the other hand, the Pictet–Spengler reaction between (±)-tryptophan ethyl ester (**12**) and 2-quinoline carbaldehyde (**13**) gave nitramarine carboxylic acid ethyl ester (**11**). Subsequent hydrolysis followed by decarboxylation gave **1**. Nitramarine carboxylic acid (**15**) and carboxamide (**16**) were found to prolong the sleeping time of mice.

Keywords——nitramarine; 1-azahexatriene; thermal cyclization; Pictet–Spengler reaction; sleeping-time prolongation

Nitramarine (**1**), 1-(2-quinolyl)-9*H*-pyrido[3,4-*b*]indole, which possesses hypnotic and hypotensive activities, was recently isolated from *Nitralia komarovii*.[1] We became interested in the synthesis of this simple β-carboline alkaloid (**1**) and its derivatives, and their prolonging effect on the sleeping time of mice.

Previously,[2] we developed a method for the synthesis of condensed heteroaromatics such as thienopyridine and γ-carboline based on thermal cyclization of an intramolecular 1-azahexatriene system. For the synthesis of nitramarine (**1**), we attempted to apply this intramolecular thermal cyclization of a 1-azahexatriene system (**2**) to the synthesis of β-carbolines as shown in the retrosynthetic pathway (Chart 1). We also examined the classical Pictet–Spengler method[3] (Chart 2) for comparison.

For the application of thermal cyclization of a 1-azahexatriene system (**2**), the vinyl oximes (**9** and **10**) were chosen as key intermediates for the synthesis of nitramarine (**1**) as shown in Chart 1. Treatment of indole-3-carbaldehyde (**3**) with dimsylsodium followed by addition of benzenesulfonyl chloride gave the *N*-benzenesulfonyl-3-indolecarbaldehyde (**4**) in 87% yield. Subsequent Wittig reaction of the aldehyde (**4**) with methylenetriphenylphosphorane or triethylphosphonoacetate afforded the *N*-benzenesulfonyl-3-vinylindole (**5**) (57%) or the α,β-unsaturated ester (**6**) (49%, only *E*-form), respectively. The vinyl indole (**5**) was treated with lithium diisopropylamide (LDA) in tetrahydrofuran at −78 °C under an N_2 stream and subsequent treatment with 2-quinolinecarbaldehyde (**13**) gave the vinyl ketone (**7**) (23%), but without any detectable amount of the expected alcohol derivative. Similarly, treatment of the α,β-unsaturated ester (**6**) with LDA followed by addition of **13** also gave only the ketone (**8**) in 23% yield.[4] The reason for the formation of the ketones is not clear at the moment.[5]

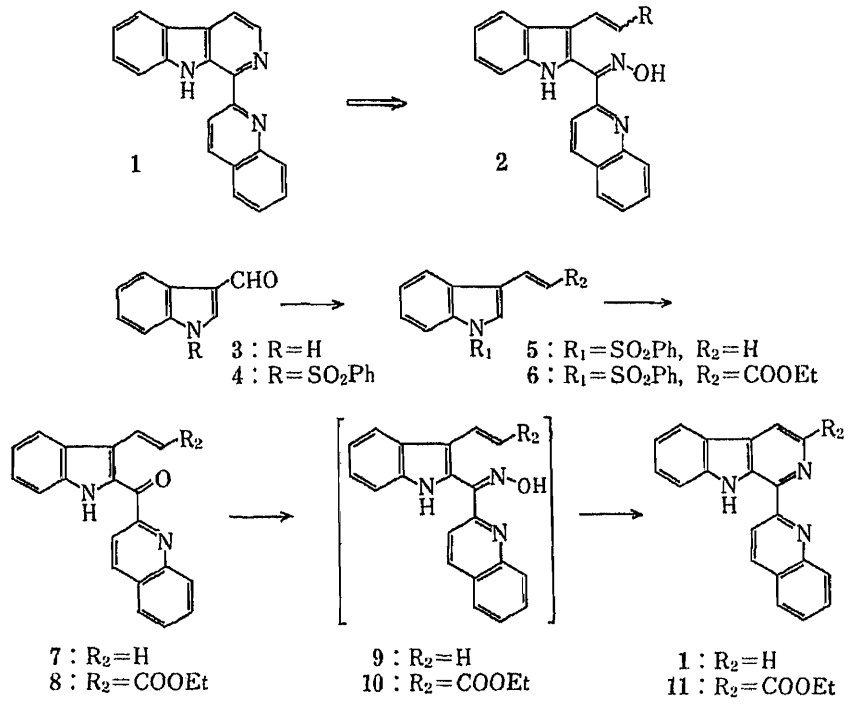

Chart 1

For the preparation of the oxime intermediate (**9**), treatment of the ketone (**7**) with hydroxylamine hydrochloride and sodium acetate in alcohol under reflux afforded only the unchanged starting material. The ketone (**7**) was then heated with hydroxylamine in toluene under reflux, giving nitramarine (**1**) in 37% yield (65% yield based on the consumed starting material), mp 180.5—182 °C from benzene (Lit.[1]; mp 172—173 °C). Spectroscopic evidence and the elemental analysis data of synthetic nitramarine (**1**) were satisfactory. The difference of melting points suggests that the synthetic nitramarine (**1**) is more pure than the natural product. On the other hand, heating of the ketone (**8**) in the presence of hydroxylamine in *o*-dichlorobenzene under reflux gave nitramarine carboxylic acid ethyl ester (**11**) (35%; 48% yield based on the consumed starting material), although other solvent such as xylene, decalin and diphenyl ether gave only poor yields of **11**.

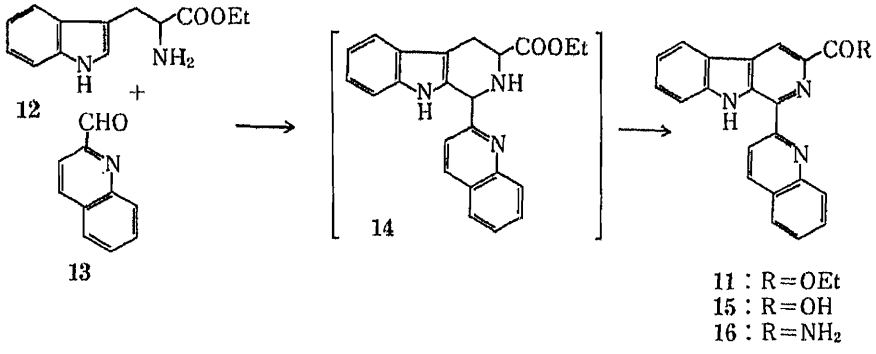

Chart 2

Alternatively, the Pictet–Spengler reaction was applied to the synthesis of nitramarine (**1**) as follows. Heating of (±)-tryptophan ethyl ester (**12**) and 2-quinolinecarbaldehyde (**13**) in benzene afforded the tetrahydro-β-carboline (**14**), which was used for the next reaction without isolation. Subsequent aromatization of **14** with 5% Pd–C in xylene gave nitramarine

TABLE I. Effects of Nitramarine (**1**), **11**, **15**, **16** and Chlorpromazine on Pentobarbital-Na Induced Sleeping Time in Mice

Compounds	mg/kg *p.o.*	Latency[a] (s)	Sleeping time[a] (s)
Control	—	224.4±15.7	2325.4±267.8
Nitramarine (**1**)	100	213.6±18.8	2594.6±240.0
The ester (**11**)	100	235.8±18.4	2782.3±143.3
The carboxylic acid (**15**)	100	209.8± 6.7	3200.0±124.5[b]
The amide (**16**)	100	249.3±23.5	3343.2±176.5[c]
Chlorpromazine	5	171.2±10.3[b]	5147.4± 31.8[d]

a) Mean±standard error (s). *b*) $p<0.05$. *c*) $p<0.01$. *d*) $p<0.001$.

carboxylic acid ethyl ester (**11**) in 63% yield. This product was identical with **11** prepared by thermal cyclization of **9**. Hydrolysis of the ethyl ester (**11**) afforded the carboxylic acid (**15**), which was decarboxylated by refluxing in quinoline to give nitramarine (**1**) quantitatively, as shown in Chart 2. The structure of the synthetic nitramarine (**1**) was also established by this alternative route. The carboxamide (**16**) was further prepared from the carboxylic acid *via* the acid chloride for pharmacological testing.

Thus, the application of thermal cyclization of a 1-azahexatriene system to the synthesis of nitramarine (**1**) was achieved, though in very poor overall yield (7.4%). It seems that the yield of the thermal cyclization mainly depends on the formation of the oxime intermediate. On the other hand, the Pictet–Spengler route gave 60% overall yield.

The prolonging effect of nitramarine and its derivatives on the sleeping time of mice was examined. As shown in Table I, among the test compounds, the carboxylic acid (**15**) and the amide (**16**) (100 mg/kg) gave sleeping time ratios of 1.38 and 1.44, respectively. On the other hand, chlorpromazine was also found to prolong the sleeping time significantly, and its sleeping time ratio was 2.21.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a Shimadzu IR-408 spectrophotometer. Ultraviolet (UV) spectra were recorded on a Shimadzu UV 240 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL PMX-60 spectrometer and chemical shifts are expressed in δ (ppm) values relative to internal Me_4Si. Mass spectra (MS) were recorded on a Shimadzu GC-MS 6020 spectrometer equipped with a direct inlet system. Elemental analysis was done with a Perkin Elmer 240-C apparatus.

***N*-Benzenesulfonyl-3-indolecarbaldehyde (4)**——A solution of indole-3-carbaldehyde (**3**) (5 g, 34.4 mmol) in dimethylsulfoxide (25 ml) under an N_2 stream was added to an ice-cooled solution of dimsylsodium prepared from 60% NaH (1.6 g, 40 mmol) and dimethylsulfoxide (15 ml) at 60 °C for 1 h. The solution was stirred at room temperature for 1 h, then a solution of benzenesulfonyl chloride (7 g, 39.6 mmol) in dimethylsulfoxide (30 ml) was added under ice-cooling. The mixture was worked up with an aqueous solution of ammonium chloride, and extracted with $CHCl_3$. The $CHCl_3$ solution was washed with brine and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography (silica gel, 100 g) with benzene to give **3** (8.5 g, 87%), mp 156—158 °C (from $CHCl_3$–hexane). IR ν_{max}^{KBr} cm^{-1}: 1672 (aldehyde). ^{1}H-NMR ($CDCl_3$) δ: 7.17—8.30 (10H, m), 10.03 (1H, s). MS *m/z*: 285 (M^+). *Anal.* Calcd for $C_{15}H_{11}NO_3S$: C, 63.15; H, 3.89; N, 4.91. Found: C, 63.20; H, 3.89; N, 4.90.

***N*-Benzenesulfonyl-3-vinylindole (5)**——A solution of *n*-BuLi in hexane (3.1 ml of 1.65 M solution in hexane, 5.1 mmol) was added dropwise to an ice-cooled solution of methyl triphenylphosphonium bromide (1.8 g, 5 mmol) in anhydrous tetrahydrofuran (THF) (20 ml) under an N_2 stream. After completion of ylid formation (about 30 min), a solution of the aldehyde (**4**) (1.5 g, 4.2 mmol) in anhydrous THF (20 ml) was added to the ylid solution and the mixture was stirred at room temperature overnight. The reaction mixture was worked up with brine, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography (silica gel, 30 g) with benzene–hexane (1 : 1, v/v) to give the

vinylindole (**5**) (0.68 g, 57% yield) as a colorless semisolid. This compound was used immediately because it is light-sensitive. ^{1}H-NMR ($CDCl_3$) δ: 5.28 (1H, dd, J=11, 2 Hz), 5.70 (1H, dd, J=18, 2 Hz), 6.72–8.02 (11H, m). MS *m/z*: 283 (M^+).

2-(3-Vinylindolyl)-2-quinolyl Ketone (7)——A solution of the vinylindole (**5**) (4.7 g, 16.6 mmol) in anhydrous THF (50 ml) was added to a solution of LDA [prepared from diisopropylamine (2.9 ml, 20.7 mmol) and *n*-BuLi in hexane (1.1 ml of 1.65 M solution in hexane, 18.1 mmol) in anhydrous THF (10 ml)] with stirring at −78 °C under an N_2 stream. After 1 h, a solution of the aldehyde (**13**) (3.14 g, 20.0 mmol) in anhydrous THF (30 ml) was added to this solution and the mixture was further stirred at −78 °C for 1 h. Then the cooling bath was removed and the mixture was stirred overnight. After being worked up with brine, the mixture was extracted with EtOAc. The extract was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel, 100 g) using EtOAc–hexane (20 : 1, v/v) to give the ketone (**7**) (1.14 g, 23% yield), mp 154—155 °C (from EtOH). IR ν_{max}^{KBr} cm^{-1}: 1680 (ketone). ^{1}H-NMR ($CDCl_3$) δ: 5.49 (1H, dd, J=11, 2 Hz), 5.88 (1H, dd, J=18, 2 Hz), 6.65—8.47 (11H, m). MS *m/z*: 298 (M^+). *Anal.* Calcd for $C_{20}H_{14}N_2O$: C, 80.51, H, 4.73; N, 9.39. Found: C, 80.50; H, 4.81; N, 9.30.

Nitramarine (1) from 7——A stirred mixture of the vinyl ketone (**6**) (100 mg, 0.336 mmol), hydroxylamine hydrochloride (234 mg, 3.36 mmol) and NaOAc (275 mg, 3.36 mmol) in toluene (10 ml) was heated at reflux temperature for 48 h. After removal of the toluene, the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by column chromatography (silica gel, 20 g). Elution with benzene provided the desired nitramarine (**1**) (36 mg, 38% yield; 65% yield based on the consumed starting material) along with unchanged starting material (36 mg). Recrystallization from benzene gave **1** as colorless needles, mp 180.5—182 °C (Lit.[1]; mp 172—173 °C). UV λ_{max}^{EtOH} nm (log ε): 230 (4.24), 242 (3.47), 272 (2.34), 306 (1.26), 385 (1.82) (Lit.[1]; 230, 246, 275, 310, 388). ^{1}H-NMR ($CDCl_3$–MeOH-d_4) δ: 7.06—8.36 (10H, m), 8.48 (1H, d, J=5 Hz), 8.78 (1H, d, J=9 Hz). MS *m/z*: 295 (M^+). *Anal.* Calcd for $C_{20}H_{13}N_3$: C, 81.33; H, 4.44; N, 14.13. Found: C, 81.40; H, 4.54; N, 14.13.

Ethyl 3-(*N*-Benzenesulfonyl-3-indolyl)acrylate (6)——A solution of the aldehyde (**4**) (5 g, 17.5 mmol) in anhydrous THF (50 ml) was added to a stirred solution of triethylphosphonoacetate (4.05 g, 21.45 mmol) and *n*-BuLi (13 ml of 1.65 M solution in hexane, 21.45 mmol) in anhydrous THF (40 ml) under cooling with ice-bath. After being stirred at room temperature for 10 h, the reaction mixture was worked up with brine and extracted with EtOAc. The EtOAc layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness. The residue was purified by column chromatography (silica gel, 60 g) with EtOAc–hexane (1 : 50, v/v) to give the ester (**6**) (3.02 g, 49% yield), mp 136—137 °C (from EtOH). IR ν_{max}^{KBr} cm^{-1}: 1710 (ester). ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, t, J=7 Hz), 4.20 (2H, q, J=7 Hz), 6.45 (1H, d, J=16 Hz), 7.03—8.03 (11H, m). MS *m/z*: 355 (M^+). *Anal.* Calcd for $C_{19}H_{17}NO_4S$: C, 64.20; H, 4.82; N, 3.94. Found: C, 64.33; H, 4.95; N, 4.10.

2-[3-(Ethyl β-Acrylyl)]indolyl-2-quinolyl Ketone (8)——A solution of the ester (**6**) (2 g, 5.63 mmol) in anhydrous THF (20 ml) was added to a solution of LDA [prepared from diisopropylamine (0.8 ml, 6.2 mmol) and *n*-BuLi (3.75 ml of 1.65 M solution in hexane, 6.19 mmol) in anhydrous THF (4 ml)] at −78 °C under an N_2 atmosphere. After 1 h, a solution of 2-quinolinecarbaldehyde (**13**) (1.06 g, 6.75 mmol) in anhydrous THF (10 ml) was added to this reaction mixture under the same conditions. Stirring was continued for 10 h and then the mixture was worked up with brine. Extraction with EtOAc gave a crude material, which was purified by column chromatography (silica gel, 30 g) with EtOAc–hexane (3 : 97, v/v) to give the ketone (**8**) (0.48 g, 23% yield) as colorless needles, mp 179.5—180 °C (from EtOH). IR ν_{max}^{KBr} cm^{-1}: 1695 (ketone). ^{1}H-NMR ($CDCl_3$) δ: 1.37 (3H, t, J=7 Hz), 4.32 (2H, q, J=7 Hz), 6.83 (1H, d, J=16 Hz), 7.03—8.33 (10H, m), 8.78 (1H, d, J=16 Hz). MS *m/z*: 370 (M^+). *Anal.* Calcd for $C_{23}H_{18}N_2O_3$: C, 74.58; H, 4.90; N, 7.56. Found: C, 74.80; H, 5.12; N, 7.65.

1-(2-Quinolyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic Acid Ethyl Ester (11)——A stirred mixture of the ketone (**8**) (100 mg, 0.27 mmol), hydroxylamine hydrochloride (190 mg, 2.73 mmol), and NaOAc (224 mg, 2.77 mmol) in *o*-dichlorobenzene (10 ml) was heated under reflux for 14 h. After removal of the solvent, the residue was extracted with $CHCl_3$. The extract was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (silica gel, 20 g) with benzene to give the ester (**11**) (35 mg, 35% yield; 48% yield based on the consumed starting material). Recrystallization from EtOH gave colorless prisms, mp 190—192 °C. IR ν_{max}^{KBr} cm^{-1}: 1725 (ester). UV λ_{max}^{EtOH} nm (log ε): 220 (3.00), 245 (3.13), 273 (3.67), 364 (1.20), 380 (1.53). ^{1}H-NMR ($CDCl_3$) δ: 1.50 (3H, t, J=7 Hz), 4.48 (2H, q, J=7 Hz), 7.03—8.30 (9H, m), 8.68 (1H, s), 8.82 (1H, d, J=8 Hz). MS *m/z*: 357 (M^+). *Anal.* Calcd for $C_{23}H_{17}N_3O_2$: C, 75.19; H, 4.66; N, 11.44. Found: C, 75.42; H, 4.77; N, 11.40.

1-(2-Quinolyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic Acid Ethyl Ester (11) from (±)-Tryptophan Ethyl Ester (12)——A stirred mixture of (±)-tryptophan ethyl ester (**12**) (10 g, 43.1 mmol) and the aldehyde (**13**) (6 g, 43.1 mmol) in benzene (200 ml) was refluxed for 14 h. The benzene was replaced by xylene (200 ml) and then 5% Pd–C (2 g) was added to the reaction mixture. This mixture was refluxed overnight. Filtration of the hot xylene solution and evaporation of the xylene gave a crude residue. This residue was recrystallized from benzene to give the β-carboline (**11**) (9.7 g, 63% yield). This compound was identical with **11** prepared by thermal cyclization.

1-(2-Quinolyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxylic Acid (15)——A mixture of the ester (**11**) (1 g, 2.8 mmol) and NaOH (0.5 g, 12.5 mmol) in water (50 ml) and EtOH (100 ml) was refluxed for 2 h. After removal of the solvent, a

solution of excess NH_4Cl in water was added. The separated crystals were filtered off, washed with water and dried, mp 259—261 °C (from EtOH). IR ν_{max}^{KBr} cm^{-1}: 1745 (carboxylic acid). MS *m/z*: 339 (M^+). *Anal.* Calcd for $C_{21}H_{13}N_3O_2$: C, 74.32; H, 3.86; N, 12.38. Found: C, 74.30; H, 3.92; N, 12.35.

Nitramarine (1) from 15——A solution of the carboxylic acid (**15**) (0.5 g, 1.47 mmol) in quinoline (50 ml) was heated under reflux temperature for 1 h, then the quinoline was removed under reduced pressure. The residue was purified by column chromatography (silica gel, 30 g) with benzene to give nitramarine (**1**) (0.43 g, 98% yield), mp 180.5—182 °C (from benzene). This synthetic nitramarine (**1**) was identical with **1** prepared by thermal cyclization.

1-(2-Quinolyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxamide (16)——A mixture of the carboxylic acid (**15**) (1 g, 2.95 mmol) and $SOCl_2$ (0.5 ml, 7.02 mmol) in benzene (100 ml) was heated under reflux for 2 h. After removal of the solvent and excess $SOCl_2$, the residue was dissolved in $CHCl_3$, and the solution was poured into aqueous ammonia (excess) with stirring. After 1 h, the precipitates were filtered off, washed with water and dried to give the amide (**16**) (0.78 g, 78% yield), mp >300 °C. IR ν_{max}^{KBr} cm^{-1}:1645 (amide). MS *m/z*: 338 (M^+). *Anal.* Calcd for $C_{21}H_{14}N_4O$: C, 74.54; H, 4.17; N, 16.56. Found: C, 74.50; H, 4.30; N, 16.44.

Pharmacology——Groups of 10 male ICR mice weighing 22 to 28 g were used, and chlorpromazine was used as a reference drug. Test compounds were administered orally 30 min before the intraperitoneal injection of pentobarbital-Na (40 mg/kg), and the sleeping time (from loss to recovery of righting reflex) was measured for each mouse. The sleeping time ratio was calculated by comparison with the sleeping time in a control group.

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Enzymes and Catalysts. I. Pig Liver Esterase-Catalyzed Hydrolysis of Heterocyclic Diesters

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The asymmetric hydrolysis of five-membered cyclic diesters was carried out by using pig liver esterase (PLE) as a catalyst. The absolute configurations of the resulting half esters and recovered diesters were established and the stereospecificity of PLE action in such systems was revealed.

Keywords——pig liver esterase; hydrolysis; diester; pyrrolidine; tetrahydrothiophene; half ester; stereochemistry

Recently, we have reported convenient methods for the synthesis of five-membered heterocycles by the use of 1,3-dipolar cycloaddition reactions.[1,2] The introduction of chirality into the resulting cycloadducts was required for further application of these methods. Asymmetric synthesis or optical resolution by the use of enzymes is an effective method for obtaining chiral synthons, and pig liver esterase (PLE) has often been used for such enzymatic synthesis[3] because it is easy to handle. We wish to describe herein the PLE-catalyzed hydrolysis of five-membered heterocyclic diesters aiming to produce chiral building blocks which are expected to be useful for the synthesis of natural products.

Eight diesters with five-membered ring systems were employed for the purposes of the present work: dimethyl pyrrolidine-3,4-dicarboxylate (**1a**), dimethyl *N*-benzylpyrrolidine-3,4-dicarboxylate (**1b**), and dimethyl tetrahydrothiophene-3,4-dicarboxylate (**1c**) were synthesized by 1,3-cycloadditions *via* azomethine or thiocarbonyl ylides. Dimethyl cyclopentane-1,2-dicarboxylate (**1d**), dimethyl pyrrolidine-2,5-dicarboxylate (**2a**), dimethyl *N*-benzyl-pyrrolidine-2,5-dicarboxylate (**2b**), and dimethyl cyclopentan-1,3-dicarboxylate (**2d**) were synthesized by the reported methods.[4-6] Dimethyl tetrahydrothiophene-2,5-dicarboxylate (**2c**) was synthesized from dimethyl 2,5-dibromoadipinate and sodium sulfide. The stereochemistry of ester groups in these compounds was restricted to *trans* from, in which the enzymatic action has not been established.

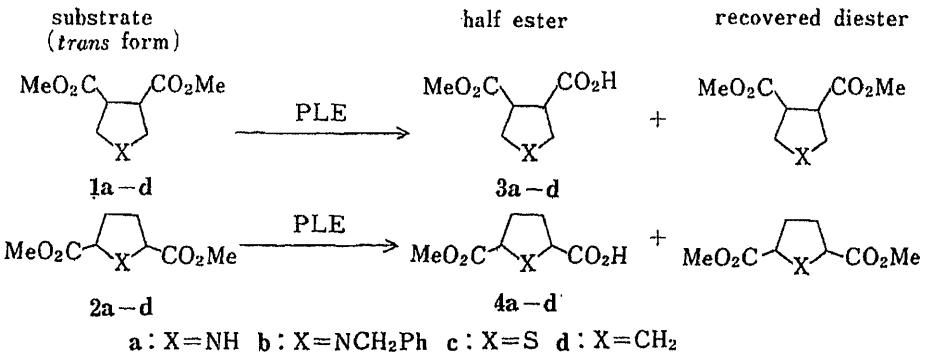
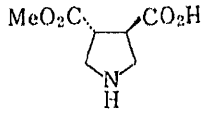
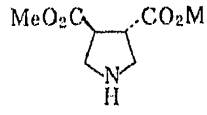
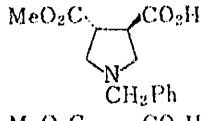
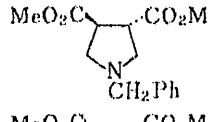
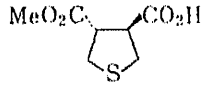
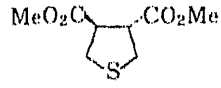
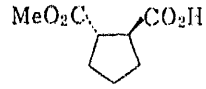
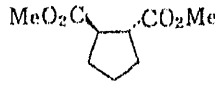
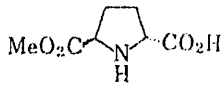
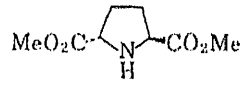
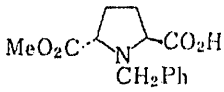
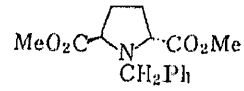
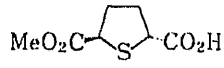
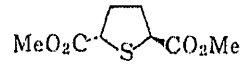
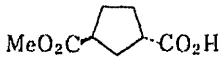
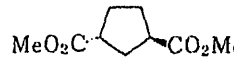


Chart 1

Treatment of diesters (**1a**—**d** and **2a**—**d**) with PLE at 25 °C in phosphate buffer solution (pH 8) gave optically active half esters and diesters, as summarized in Table I. The enantiomeric excess (% ee) of each product was determined by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analysis using tris-[3-(heptafluoropropyl-hydroxy-methylene)-*d*-camphorato] europium(III) derivative or tris-[3-(trifluoro-methylhydroxy-methylene)-*d*-camphorato] europium(III) derivative as a shift reagent; this was done after methylation with diazomethane in the case of the half esters (**3a**—**d** and **4a**—**d**). The absolute configurations were established by chemical transformations into products which could be compared with authentic specimens.

Reduction of (−)-**1b** with $LiAlH_4$ followed by treatment with *p*-toluenesulfonyl chloride and reduction of the resulting product with $LiAlH_4$ gave (−)-*N*-benzyl-3,4-dimethyl-pyrrolidine ((−)-**7**), which was subjected to catalytic hydrogenation over Pd–C and treated with *p*-toluenesulfonic acid to give (−)-3,4-dimethylpyrrolidine tosylate ((−)-**8**). The absolute configuration of (−)-**8** has been established as 3*S*, 4*S* by McCasland and Proskow.[7] The configuration of (−)-**1a** in entry 1 was determined by its transformation into (−)-**1b**. The optically active compounds (−)-**2a** and (−)-**2b** were identified by comparing their optical rotations with those of samples derived from authentic (−)-*N*-benzyl-pyrrolidine-2,5-dicarboxylic acid (2*S*, 5*S*).[8] (+)-Dimethyl tetrahydrothiophene-3,4-

TABLE I. PLE-Catalyzed Hydrolysis of Five-Membered Cyclic Diesters

Entry	Substrate	Half ester			Recovered diester	
		Chemical yield (%)	Optical yield (%)		Chemical yield (%)	Optical yield (%)
1	**1a**		66	23 (*R*,*R*)		64 (*S*,*S*)
2	**1b**		34	51 (*R*,*R*)		11 (*S*,*S*)
3	**1c**		33	29 (*S*,*S*)		42 (*R*,*R*)
4	**1d**		43	59 (*S*,*S*)		49 (*R*,*R*)
5	**2a**		71	10 (*R*,*R*)		11 (*S*,*S*)
6	**2b**		54	23 (*S*,*S*)		28 (*R*,*R*)
7	**2c**		48	20 (*R*,*R*)		30 (*S*,*S*)
8	**2d**		53	5 (*S*,*S*)		7 (*R*,*R*)

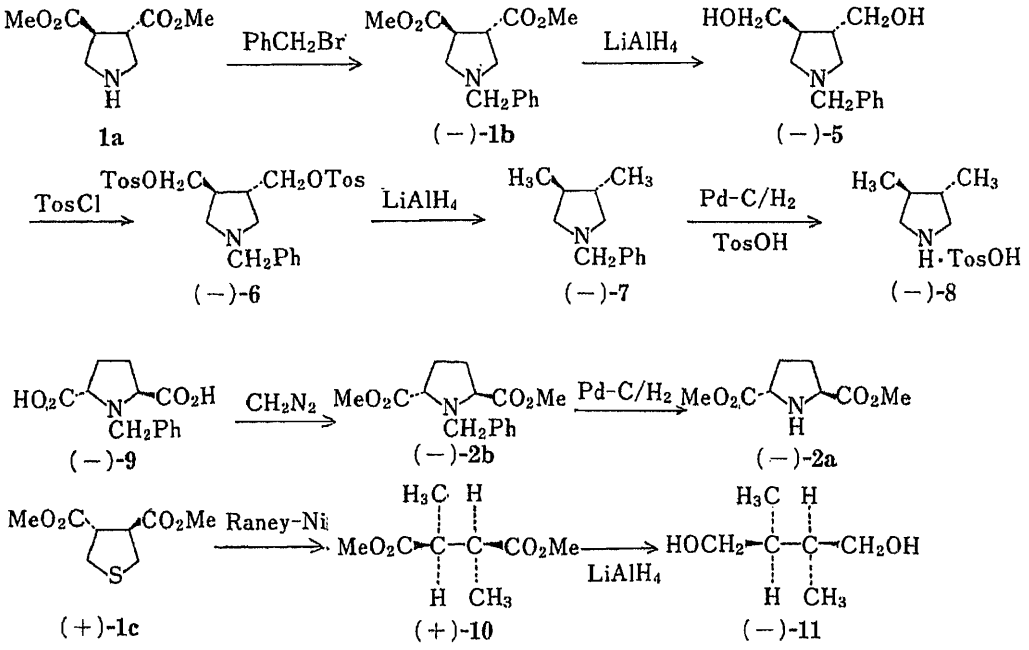


Chart 2

dicarboxylate ((+)-**1c**) was desulfurized with Raney Ni and reduced with $LiAlH_4$ to give (−)-2,3-dimethyl-1,4-butanediol ((−)-**11**), which has been established to have 2*S*, 3*S* configulation.[7] The configurations of (−)-**1d**, (−)-**2c**, and (−)-**2d** were determined readily by conversion of these compounds into the corresponding dicarboxylic acids (1*R* 2*R*,[9] 2*R* 5*R*,[10] and 1*R* 3*R*,[6a] respectively).

Table I shows that in a series of 3,4- and 1,2-diesters (entries 1—4), enantiomers of the same absolute configuration (independent of the description *R* and *S*) were hydrolyzed preferentially. In a series of 2,5- and 1,3-diesters (entries 5—8), the enantiomers hydrolyzed preferentially in the cases of entries 5, 7, and 8 were of the same absolute configuration. However, in the case of entry 6 the reverse configuration was favored.

The PLE-catalyzed hydrolysis of five-membered cyclic diesters proceeded smoothly to give optically active half esters and diesters. Although the optical yields (% ee) were relatively low, it should be noted that the stereospecificity of PLE was not influenced by the hetero atom, but was influenced by a substituent adjacent to a chiral center.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO A 202 infrared spectrophotometer. ^{1}H-NMR spectra were determined with a Hitachi R-24B (60 MHz) NMR spectrometer or a JEOL 90Q (90 MHz) FT-NMR spectrometer. Chemical shifts were measured relative to internal tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt hydrate, and are given in δ values. Coupling constants are reported in hertz and splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Optical rotations were measured with a JASCO DIP-140 digital polarimeter.

Starting Materials——Dimethyl *N*-benzylpyrrolidine-3,4-dicarboxylate (**1b**),[1] dimethyl *N*-benzylpyrrolidine-2,5-dicarboxylate (**2b**),[4] dimethyl tetrahydrothiophene-3,4-dicarboxylate (**1c**),[2] dimethyl cyclopentane-1,2-dicarboxylate (**1d**),[5] dimethyl cyclopentane-1,3-dicarboxylate (**2d**)[6] were prepared by the reported methods.

Dimethyl Pyrrolidine-3,4-dicarboxylate (1a)——A mixture of **1b** (1.39 g, 5 mmol) and 5% Pd–C (0.4 g) in methanol (50 ml) was stirred overnight at room temperature under a hydrogen atmosphere. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to give an oil, 0.86 g (92%). IR ν_{max}^{neat} cm^{-1}: 1736 (C=O), 3328 (NH). ^{1}H-NMR ($CDCl_3$) δ: 2.10 (1H, s, NH), 2.40—3.36 (6H, m, 2×NCH_2CH), 3.71 (6H, s, 2×OCH_3). High-resolution MS (*m/z*): Calcd for $C_8H_{13}NO_4$ (M^+): 187.0845. Found: 187.0805.

Dimethyl Pyrrolidine-2,5-dicarboxylate (2a)——A mixture of **2b** (1.46 g, 5.27 mmol) and 5% Pd–C (0.7 g) in methanol (50 ml) was stirred for 2 d at room temperature under a hydrogen atmosphere. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to give an oil, 0.77 g (78%). IR ν_{max}^{neat} cm^{-1}: 1737 (C=O), 3368 (NH). ^{1}H-NMR ($CDCl_3$) δ: 1.75—2.39 (4H, m, 2×$CHC\underline{H}_2$), 2.68 (1H, br, NH), 3.74 (6H, s, 2×OCH_3), 3.90—4.07 (2H, m, 2×$C\underline{H}CH_2$). High-resolution MS (*m/z*): Calcd for $C_8H_{13}NO_4$ (M^+): 187.0845. Found: 187.0826.

Dimethyl Tetrahydrothiophene-2,5-dicarboxylate (2c)——A solution of dimethyl 2,5-dibromoadipinate (10.0 g, 30 mmol) in benzene (6 ml) was added dropwise to a solution of sodium sulfide nonahydrate (7.2 g, 30 mmol) and tetra-*n*-butylammonium bromide (100 mg) in H_2O (6 ml), and the suspension was stirred at room temperature for 5 h. The organic layer was separated and the aqueous layer was extracted with benzene. The combined benzene solution was dried over $MgSO_4$ and concentrated under reduced pressure. Silica gel column chromatography of the residue with $CHCl_3$–benzene (1 : 1) gave an oil, 1.6 g (26%). IR ν_{max}^{neat} cm^{-1}: 1740 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 2.25—2.49 (4H, m, 2×CH_2), 3.72 (6H, s, 2×CH_3), 3.96—4.05 (2H, m, 2×CH). High-resolution MS (*m/z*): Calcd for $C_8H_{12}O_4S$ (M^+): 204.0456. Found: 204.0428. Its stereochemistry (2,5-*trans*) was supported by the fact that the isolable *cis* isomer was converted into **2c** by treatment with sodium methoxide in methanol.

General Procedure for PLE-Catalyzed Hydrolysis of 1a—b and 2a—b——A mixture of PLE (200 unit) and a diester (2 mmol) (**1a—b** and **2a—b**) in 0.1 M phosphate buffer of pH 8.0 (20 ml) was stirred vigorously at 25 °C. After 8—30 min, the pH was adjusted to 10 and the mixture was extracted with ether or hexane. The organic layer was washed with sat. NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to give an optically active diester. The aqueous layer was neutralized with 3 N HCl, and concentrated under reduced pressure. Then $CHCl_3$ or methanol was added to the residue. After removal of insoluble material by filtration, concentration of the filtrate gave a half ester (**3a—b** and **4a—b**), which was characterized on the basis of the ^{1}H-NMR spectrum and treated with diazomethane to afford the corresponding diester. Chemical and optical yields of the products are listed in Table I and the optical rotations are given below. In entries 1, 2, 5, and 6, the half esters **3a**, **3b**, **4a**, and **4b** were converted into (+)-**1a**, (+)-**1b**, (+)-**2a**, and (−)-**2b**, respectively. Entry 1: (+)-**1a** $[\alpha]_D^{23}$ +37.00° (*c*=1.20, $CHCl_3$), (−)-**1a** $[\alpha]_D^{23}$ −102.14° (*c*=1.40, $CHCl_3$). Entry 2: (+)-**1b** $[\alpha]_D^{25}$ +13.77° (*c*=1.22, $CHCl_3$), (−)-**1b** $[\alpha]_D^{26}$ −5.94° (*c*=2.00, $CHCl_3$). Entry 5: (+)-**2a** $[\alpha]_D^{19}$ +4.67° (*c*=3.08, MeOH), (−)-**2a** $[\alpha]_D^{19}$ −5.20° (*c*=2.04, MeOH). Entry 6: (−)-**2b** $[\alpha]_D^{25}$ −19.02° (*c*=2.24, $CHCl_3$), (+)-**2b** $[\alpha]_D^{24}$ +28.80° (*c*=2.00, $CHCl_3$). ^{1}H-NMR (solvent) δ: **3a** (D_2O), 3.49—3.75 (6H, m, 2×CH_2CH), 3.77 (3H, s, CH_3). **3b** ($CDCl_3$), 2.88—4.53 (8H, m, 2×CH_2CH, NCH_2Ph), 3.69 (3H, s, CH_3), 7.36 (5H, s, C_6H_5), 9.22—9.64 (1H, br, OH). **3c** ($CDCl_3$), 3.00—3.35 (4H, m, 2×CH_2), 3.35—3.65 (2H, m, 2×CH), 3.70 (3H, s, CH_3), 8.37—8.64 (1H, br, OH). **3d** ($CDCl_3$), 1.63—2.22 (6H, m, 3×CH_2), 3.01—3.26 (2H, m, 2×CH), 3.70 (3H, s, CH_3), 9.80—10.28 (1H, br, OH).

General Procedure for PLE-Catalyzed Hydrolysis of 1c—d and 2c—d——A mixture of PLE (200 unit) and a dimethylester (2 mmol) (**1c—d** and **2c—d**) in 0.1 M phosphate buffer of pH 8.0 (20 ml) was stirred vigorously at 25 °C. The pH value was maintained at 8 by the addition of 1 N NaOH solution. After 4—15 min, the pH value was adjusted to 10 and the mixture was extracted with ether or CH_2Cl_2. The organic layer was washed with sat. NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to give an optically active diester. The aqueous layer was acidified to pH 2 with 3 N HCl and extracted with ether or CH_2Cl_2. The organic layer was washed with sat. NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to give a half ester (**3c—d** and **4c—d**), which was characterized on the basis of the ^{1}H-NMR spectrum and treated with diazomethane to afford the corresponding diester. Chemical and optical yields of the products are listed in Table I and the optical rotations are given below. In entries 3, 4, 7, and 8, the half esters **3c**, **3d**, **4c**, and **4d** were converted into (+)-**1c**, (+)-**1d**, (−)-**2c**, and (+)-**2d**, respectively. Entry 3: (+)-**1c** $[\alpha]_D^{27}$ +14.71° (*c*=0.90, $CHCl_3$), (−)-**1c** $[\alpha]_D^{25}$ −20.86° (*c*=1.94, $CHCl_3$). Entry 4: (+)-**1d** $[\alpha]_D^{20}$ +49.60° (*c*=1.00, $CHCl_3$), (−)-**1d** $[\alpha]_D^{20}$ −41.29° (*c*=2.02, $CHCl_3$). Entry 7: (−)-**2c** $[\alpha]_D^{23}$ −17.27° (*c*=3.52, $CHCl_3$), (+)-**2c** $[\alpha]_D^{23}$ +26.29° (*c*=1.94, $CHCl_3$). Entry 8: (+)-**2d** $[\alpha]_D^{20}$ +3.06° (*c*=1.58, $CHCl_3$), (−)-**2d** $[\alpha]_D^{20}$ −1.52° (*c*=1.98, $CHCl_3$). ^{1}H-NMR (solvent) δ: **4a** (D_2O), 2.01—2.68 (4H, m, 2×CH_2), 3.85 (3H, s, CH_3), 4.16—4.62 (2H, m, 2×CH). **4b** ($CDCl_3$), 1.80—2.67 (4H, m, CH_2CH_2), 3.69 (3H, s, CH_3), 3.64—3.85 (2H, m, 2×CH), 3.92 (2H, s, NCH_2), 7.29 (5H, s, C_6H_5), 9.30—9.75 (1H, br, OH). **4c** ($CDCl_3$), 2.12—2.69 (4H, m, 2×CH_2), 3.72 (3H, s, CH_3), 3.97—4.23 (2H, m, 2×CH), 7.63 (1H, s, OH). **4d** ($CDCl_3$), 1.65—2.31 (6H, m, 3×CH_2), 2.52—3.05 (2H, m, 2×CH), 3.68 (3H, s, CH_3), 9.81 (1H, s, OH).

(−)-Dimethyl *N*-Benzylpyrrolidine-3,4-dicarboxylate ((−)-1b)——A mixture of (−)-**1a** (70 mg, 0.37 mmol) ($[\alpha]_D^{23}$ −102.14°, *c*=1.40, $CHCl_3$), benzyl bromide (77 mg, 0.45 mmol), and triethylamine (46 mg, 0.45 mmol) in dry tetrahydrofuran (THF) (5 ml) was stirred at room temperature. After 20 h, the insoluble material was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by thin layer chromatography ($CHCl_3$: THF = 60 : 1) to give an oil, 78 mg (76%). $[\alpha]_D^{19}$ −17.31° (*c*=1.56, $CHCl_3$). The spectral data of this compound were in agreement with that of **1b**.

(−)-*N*-Benzylpyrrolidine-3,4-dimethanol ((−)-5)——A solution of (−)-**1b** (641 mg, 2.3 mmol) ($[\alpha]_D^{21}$ −12.28°, *c*=3.16, $CHCl_3$, 45% ee) in dry ether (10 ml) was added dropwise to a suspension of $LiAlH_4$ (137 mg, 3.6 mmol) in dry ether (10 ml) in an ice-water bath, and the ethereal solution was refluxed for 2 h, then allowed to cool. A 5% NaOH solution (0.3 ml) was added to the mixture and insoluble material was filtered off. The filtrate was concentrated under

reduced pressure to give a solid, 374 mg (74%). $[\alpha]_D^{20}$ −16.70° (c=2.00, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3384 (OH). ^{1}H-NMR ($CDCl_3$) δ: 2.02—2.46 (6H, m, 2×NC$\underline{H}_2$CH, 2×O$\underline{H}$), 2.66—2.86 (2H, m, 2×CH$_2$C$\underline{H}$), 3.57 (2H, s, NC$\underline{H}_2$Ph), 3.61 (4H, d, J=4.6 Hz, 2×C$\underline{H}_2$OH), 7.26 (5H, s, C_6H_5). *Anal.* Calcd for $C_{13}H_{19}NO_2$: C, 70.56; H, 8.65; N, 6.33. Found: C, 70.61; H, 8.58; N, 6.39.

(−)-*N*-Benzyl-3,4-bis(tosyloxymethyl)pyrrolidine ((−)-6)——A mixture of (−)-**5** (374 mg, 1.7 mmol) and tosyl chloride (972 mg, 5.1 mmol) in pyridine (5 ml) was stirred at room temperature. After 5 h, the reaction was quenched with water and the mixture was extracted with CH_2Cl_2. The organic layer was washed with sat. $NaHCO_3$ and water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with hexane–$CHCl_3$ (1 : 2) to give a solid, 811 mg (90%). $[\alpha]_D^{21}$ −8.47° (c=6.00, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 1175, 1360 (SO_2). ^{1}H-NMR ($CDCl_3$) δ: 1.95—2.77 (6H, m, 2×C$\underline{H}$CH$_2$), 2.44 (6H, s, 2×CH$_3$), 3.47 (2H, s, C$\underline{H}_2$Ph), 3.92 (4H, d, J=6.1 Hz, 2×CH$_2$O), 7.23 (5H, s, C_6H_5), 7.33, 7.75 (8H, AB type d's, J=8.3 Hz, 2×C_6H_4). *Anal.* Calcd for $C_{27}H_{31}NO_6S_2$: C, 61.23; H, 5.90; N, 2.64. Found: C, 61.14; H, 5.88; N, 2.59.

(−)-*N*-Benzyl-3,4-dimethylpyrrolidine ((−)-7)——A solution of (−)-**6** (811 mg, 1.53 mmol) in THF (20 ml) was added dropwise to a suspension of $LiAlH_4$ (426 mg, 11.2 mmol) in dry ether (30 ml) in an ice-water bath, and the mixture was refluxed for 3 h, then allowed to cool. A 5% NaOH solution (0.2 ml) was added to the mixture, and insoluble material was filtered off and washed with ether. The combined ethereal solution was dried over K_2CO_3, and concentrated under reduced pressure, and the residue was purified by distillation to give an oil, 217 mg (75%), bp 105 °C (bath temp.) (0.2 mmHg). $[\alpha]_D^{21}$ −21.90° (c=2.00, $CHCl_3$). IR ν_{max}^{neat} cm^{-1}: 2960, 2916, 2792 (alkyl). ^{1}H-NMR ($CDCl_3$) δ: 0.99 (6H, d, J=5.4 Hz, 2×CH$_3$), 1.54—1.87 (2H, m, 2×CH$_2$C$\underline{H}$), 2.24 (2H, t-like, 2×NC$\underline{H}_A$H), 2.76 (2H, t-like, 2×NCH$\underline{H}_B$), 3.59, 3.55 (2H, AB type d's, J=12.8 Hz, NC$\underline{H}_2$Ph), 7.27 (5H, s, C_6H_5). This compound was used in the synthesis of (−)-**8**.

(−)-3,4-Dimethylpyrrolidine Tosylate ((−)-8)——A mixture of (−)-**7** (217 mg, 1.15 mmol) and 5% Pd–C (50 mg) in methanol (8 ml) was stirred overnight at room temperature under a hydrogen atmosphere. After removal of the catalyst by filtration, the filtrate was concentrated to give an oily residue, 46 mg. This residue was treated with *p*-toluenesulfonic acid (88 mg, 0.46 mmol) to give colorless crystals, 33 mg (10%), mp 139—140 °C. $[\alpha]_D^{20}$ −7.3° (c=0.26, MeOH) [lit,[7] 3*R*, 4*R*-(+)-**8**, $[\alpha]_D^{21}$ +17.5° (c=2, MeOH)]. IR ν_{max}^{KBr} cm^{-1}: 1120 (SO_2), 3436 (NH). *Anal.* Calcd for $C_{13}H_{21}NO_3S$: C, 57.54; H, 7.80; N, 5.16. Found: C, 57.40; H, 7.73; N, 5.15.

(−)-Dimethyl *N*-Benzylpyrrolidine-2,5-dicarboxylate ((−)-2b)——(−)-*N*-Benzylpyrrolidine-2,5-dicarboxylic acid ((−)-**9**) (27 mg, 0.11 mmol) ($[\alpha]_D^{20}$ −44.81°, c=0.54, H_2O) [lit,[8] 2*R*, 5*R*-(+)-**9**, $[\alpha]_D^{25}$ +93° (c=1.00, H_2O)] was suspended in ethereal diazomethane overnight. The suspension was concentrated under reduced pressure, and the residue was purified on silica gel column chromatography with $CHCl_3$ to give an oil, 30 mg (100%). $[\alpha]_D^{19}$ −39.67° (c=0.60, $CHCl_3$). The spectral data of this compound were in agreement with those of **2b**.

(−)-Dimethyl Pyrrolidine-2,5-dicarboxylate ((−)-2a)——A mixture of (−)-**2b** (30 mg, 0.11 mmol) and 5% Pd–C (15 mg) in methanol (3 ml) was treated as described for the preparation of **2a** to give an oil, 18 mg (87%). $[\alpha]_D^{19}$ −22.78° (c=0.36, MeOH). The spectral data of this compound were in agreement with those of **2a**.

(+)-Dimethyl 2,3-Dimethylsuccinate ((+)-10)——A mixture of (+)-**1c** (607 mg, 3.13 mmol) ($[\alpha]_D^{23}$ +9.13°, c=6.00, $CHCl_3$, 18% ee) and Raney Ni in methanol (30 ml) was stirred for 20 min at room temperature. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure, and the residue was purified by distillation to give a colorless oil, 319 mg (62%), bp 115 °C (bath temp.) (22 mmHg). $[\alpha]_D^{23}$ +0.63° (c=6.0, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.09—1.30 (6H, m, 2×CH$_3$), 2.60—3.00 (2H, m, 2×CH), 3.66 (6H, s, 2×OCH$_3$). This compound was used for the synthesis of (−)-**11**.

(−)-2,3-Dimethylbutanediol ((−)-11)——A solution of (+)-**10** (319 mg, 1.92 mmol) in ether (5 ml) was added dropwise to a suspension of $LiAlH_4$ (111 mg, 2.92 mmol) in ether (5 ml) in an ice-water bath, and the mixture was refluxed for 5 h, then allowed to cool. A 5% NaOH solution was added to the mixture, and insoluble material was filtered off and washed with ether. The combined ethereal solution was concentrated under reduced pressure. The residue was purified by distillation to give a colorless oil, 218 mg (95%), bp 140 °C (bath temp.) (10 mmHg). $[\alpha]_D^{23}$ −0.33° (c=7.79, ether) [lit,[7] 2*S*, 3*S*-(−)-**11**, $[\alpha]_D^{25}$ −5.42° (c=5, ether)]. IR ν_{max}^{neat} cm^{-1}: 3320 (OH). ^{1}H-NMR ($CDCl_3$) δ: 0.87 (6H, d, J=6.0 Hz, 2×CH$_3$), 1.44—2.00 (2H, m, 2×CH), 2.20—3.20 (2H, br, 2×OH), 3.42—3.62 (4H, m, 2×CH$_2$O). High-resolution MS (m/z): Calcd for $C_6H_4O_2$ (M^+): 118.0994. Found 118.0928.

(−)-Tetrahydrothiophene-2,5-dicarboxylic Acid ((−)-12)——A mixture of (−)-**2c** (176 mg, 0.86 mmol) and conc. HCl (2 ml) was stirred at 60—70 °C for 5 h. After removal of the hydrochloric acid, the residue was diluted with water. The pH of the mixture was adjusted to 10 with 1 N NaOH and the solution was washed with $CHCl_3$. The aqueous layer was acidified to pH 2 and extracted with ether. The ethereal solution was dried over $MgSO_4$, and concentrated under reduced pressure to give crystals, 115 mg (76%), mp 148—149 °C. $[\alpha]_D^{21}$ −18.20° (c=2.00, EtOH) [lit,[10] 2*S*, 5*S*-(+)-**12**, $[\alpha]_D^{25}$ +258.5° (EtOH)]. IR ν_{max}^{KBr} cm^{-1}: 1699 (C=O), 3050 (OH). ^{1}H-NMR (D_2O) δ: 2.25—2.41 (4H, m, 2×CHC$\underline{H}_2$), 4.12—4.39 (2H, m, 2×C$\underline{H}$CH$_2$). *Anal.* Calcd for $C_6H_8O_4S$: C, 40.90; H, 4.58. Found: C, 40.95; H, 4.58.

(−)-Cyclopentane-1,2-dicarboxylic Acid ((−)-13)——A mixture of (−)-**1d** (101 mg, 0.54 mmol) and 10% HCl solution (2 ml) was stirred at 80 °C for 2 h, then allowed to cool. The precipitate was filtered off and washed with a small amount of water, 62 mg (73%), mp 161—162 °C. $[\alpha]_D^{20}$ −55.25° (c=0.80, MeOH) [lit,[9] 1*R*, 2*R*-(−)-**13**, $[\alpha]_D^{25}$ −85.9° (MeOH)]. IR ν_{max}^{KBr} cm^{-1}: 1705 (C=O), 3040 (OH). ^{1}H-NMR (D_2O) δ: 1.54—2.26 (6H, m, $CH_2CH_2CH_2$),

2.80—3.25 (2H, m, CHCH). *Anal.* Calcd for $C_7H_{10}O_4$: C, 53.16; H, 6.37. Found: C, 53.38; H, 6.44.

(−)-Cyclopentane-1,3-dicarboxylic Acid ((−)-14)——(−)-**2d** (198 mg, 1.06 mmol) was hydrolyzed in the same manner as described above for the synthesis of (−)-**13** to give crystals, 146 mg (87%), mp 77—78 °C. $[\alpha]_D^{20}$ −1.78° (c=1.46, H_2O) [lit,[6a] 1*S*, 3*S*-(+)-**14**, $[\alpha]_D^{20}$ +35.6° (c=8, H_2O)]. IR ν_{max}^{KBr} cm^{-1}: 1704 (C=O), 3050 (OH). ^{1}H-NMR (D_2O) δ: 1.68—2.43 (6H, m, $CHC\underline{H}_2C\underline{H}_2CHC\underline{H}_2$), 2.75—3.15 (2H, m, $C\underline{H}CH_2CH_2C\underline{H}CH_2$). *Anal.* Calcd for $C_7H_{10}O_4$: C, 53.16; H, 6.37. Found: C, 53.27; H, 6.36.

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Studies on the Sesquiterpenes from *Ambrosia elatior* LINNÉ[1)]

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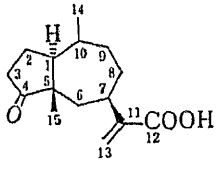
Two new sesquiterpenes, 6α-hydroxyeudesm-4(15)-ene-9β-*O*-anisate (**3**) and 1β-hydroxyeudesma-4,11(13)-dien-12-oic acid (**4**), were isolated, together with damsinic acid (**1**) and 1β,6α-dihydroxyeudesm-4(15)-ene (**2**), from *Ambrosia elatior* (Compositae). The structures were determined by chemical and spectroscopic methods. ^1H–^1H, ^1H–^{13}C and long-range ^1H–^{13}C two dimensional chemical correlation nuclear magnetic resonance analyses were carried out to confirm the structure of **4**.

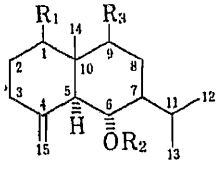
Keywords——*Ambrosia elatior*; Compositae; sesquiterpene; 6α-hydroxyeudesm-4(15)-ene-9β-*O*-anisate; 1β-hydroxyeudesma-4,11(13)-dien-12-oic acid; damsinic acid; 1β,6α-dihydroxyeudesm-4(15)-ene; ^1H-NMR; ^{13}C-NMR; 2D-NMR

In our previous papers,[2,3)] we reported the isolation of sterols and ambrosic acid[2,4)] from the pollen grains of *Ambrosia elatior* LINNÉ (Japanese name "butakusa", Compositae) or *A. artemisiaefolia* LINNÉ[4)] (Compositae). This paper reports the structural elucidation of two new sesquiterpenes, **3** and **4**, from the aerial parts of *A. elatior*. The aerial parts were extracted with methanol and the extract was partitioned with ether to obtain acidic, phenolic and neutral fractions. Repeated chromatography of the acidic and neutral fractions gave compounds **1** and **4**, and compounds **2** and **3**, respectively, whose structures were elucidated on the basis of spectroscopic data and chemical evidence.

Compound **1** was obtained as colorless needles of mp 120—125 °C, and its formula, $C_{15}H_{22}O_3$, was confirmed by high-resolution mass (HR-MS) spectroscopy. The infrared (IR) spectrum indicated the presence of an α,β-unsaturated acid function (3400 and 1704 cm^{-1}) and a cyclopentanone moiety (1724 cm^{-1}). The proton nuclear magnetic resonance (^1H-



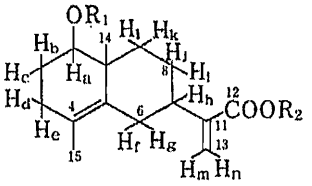
1



2 : $R_1=OH$, $R_2=R_3=H$
2a: $R_1=OCOCH_3$, $R_2=R_3=H$
2b: $R_1=OCOCH_3$, $R_2=COCH_3$, $R_3=H$

3 : $R_1=R_2=H$, $R_3=OOC$–(7′,1′,6′)–OCH_3

3a: $R_1=R_2=H$, $R_3=OH$



4 : $R_1=R_2=H$
4a: $R_1=COCH_3$, $R_2=H$
4b: $R_1=H$, $R_2=CH_3$

Chart 1

NMR) spectrum showed two methyl signals at δ 0.95 (3H, d, J=7.3 Hz) and 1.00 (3H, s), and exomethylene protons at δ 5.62 and 6.43 (each 1H, brs). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum was similar to that of ambrosic acid[4] except for the signals of C-1 and C-8, which were shifted upfield to δ 46.18 (Δ −43.62 ppm) and 31.47 (Δ −43.13 ppm), suggesting that the epoxide between C-1 and C-8 was removed.

Compound **1** was established to be damsinic acid[5] from the spectral data (^{1}H-, ^{13}C-NMR and MS).

Compound **2** was obtained as colorless needles of mp 124—125 °C, and its formula, $C_{15}H_{26}O_2$, was confirmed by HR-MS spectroscopy. The IR spectrum showed hydroxyl (3260 cm^{-1}) and double bond (1618 cm^{-1}) absorptions. The ^{1}H-NMR spectrum showed three methyl signals at δ 0.69 (3H, s), 0.86 (3H, d, J=7.0 Hz) and 0.95 (3H, d, J=7.0 Hz). The broadened methine proton (δ 1.73) at C-5 was coupled with vinyl protons (δ 4.73 and 5.01). Acetylation of **2** by the conventional method gave a monoacetate **2a** (δ 2.03, 3H, s) and a diacetate **2b** (δ 1.99 and 2.04, each 3H, s), suggesting steric hindrance at one of two hydroxyl groups.

A ^{1}H-NMR spin decoupling study allowed the assignment of all signals. Moreover, nuclear Overhauser effect (NOE) difference spectra further supported the proposed stereochemistry. Compound **2** was concluded to be identical with 1β,6α-dihydroxyeudesm-4(15)-ene from a comparison of the IR and ^{1}H-NMR spectra with reported data.[6]

Compound **3** was obtained as colorless needles of mp 191—192 °C, and its formula, $C_{23}H_{32}O_4$, was determined by HR-MS. The IR spectrum showed hydroxyl (3475 cm^{-1}), ester (1710 and 1200 cm^{-1}) and aromatic (1610, 1510, 1455 and 770 cm^{-1}) absorptions. The ^{1}H-NMR spectrum exhibited three methyl signals at δ 0.95 (3H, s), 0.87 (3H, d, J=7.0 Hz) and 0.95 (3H, d, J=7.0 Hz), two methine protons at δ 3.70 (1H, dd, J=10.0, 10.0 Hz) and 4.95

TABLE I. ^{13}C-NMR Chemical Shifts of **2**, **3** and **3a**

Carbon No.	2	3	3a
1	78.90	37.78	37.74
2	31.89	23.60	23.82
3	35.11	37.61	37.90
4	146.21	146.86	147.39
5	55.84	56.59	56.63
6	67.03	66.36	66.52
7	49.32	46.99	47.16
8	18.12	24.15	27.28
9	36.28	80.45	42.21
10	41.68	41.55	42.21
11	25.94	25.96	25.87
12	16.17	16.12	16.09
13	21.10	21.07	21.10
14	11.58	12.69	11.34
15	107.77	107.88	107.39
1′		123.03	
2′		131.56	
3′		113.63	
4′		163.36	
5′		113.63	
6′		131.56	
7′		165.88	
$\underline{CH_3O}$–		55.45	

Measured in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard.

TABLE II. ^{1}H-NMR Chemical Shifts and Coupling Constants of **3** and **3a**

Proton No.		**3**	**3a**
1	α	1.32 (1H, ddd, J=4.2, 12.8, 13.0 Hz)	
3	β	2.36 (1H, ddd, J=1.8, 3.7, 12.8 Hz)	2.35 (1H, ddd, J=1.8, 3.7, 12.8 Hz)
5	α	2.00 (1H, brd, J=10.0 Hz)	
6	β	3.70 (1H, dd, J=10.0, 10.0 Hz)	3.63 (1H, dd, J=10.0, 10.0 Hz)
7	α	1.55 (1H, dddd, J=2.8, 3.4, 10.0, 12.6 Hz)	
8	α	1.82 (1H, ddd, J=3.4, 4.1, 12.5 Hz)	
	β	1.46 (1H, ddd, J=11.3, 12.5, 12.6 Hz)	
9	α	4.95 (1H, dd, J=4.1, 11.3 Hz)	3.48 (1H, dd, J=4.1, 11.3 Hz)
11		2.28 (1H, sept d, J=2.8, 7.0 Hz)	2.23 (1H, sept d, J=2.8, 7.0 Hz)
12		0.89 (3H, d, J=7.0 Hz)	0.87 (3H, d, J=7.0 Hz)
13		0.95 (3H, d, J=7.0 Hz)	0.96 (3H, d, J=7.0 Hz)
14		0.95 (3H, s)	0.74 (3H, s)
15		4.76 (1H, bd s)	4.70 (1H, br s)
		5.08 (1H, br s)	5.05 (1H, br s)
2′,6′		7.98 (2H, dd, J=2.1, 6.7 Hz)	
3′,5′		6.92 (2H, dd, J=2.1, 6.7 Hz)	
$\underline{CH_3}O$–		3.860 (3H, s)	

Measured in $CDCl_3$ with TMS as an internal standard.

(1H, dd, J=4.1, 11.3 Hz), a methoxyl signal at δ 3.86 (3H, s), and aromatic proton signals (δ 6.69, 7.98, each 2H, dd, J=2.1, 6.7 Hz). The ^{13}C-NMR spectrum was similar to that of **2** except for an ester moiety, which consists of an ester carbonyl (δ 165.88, s), four methines (δ 113.63×2, d; 131.56×2, d), and two quaternary carbons (δ 123.03, s; 131.56, s), one of which carries a methoxyl group (δ 55.45, q).

The HR-MS of **3** showed the base ion peak at m/z 135.0495, corresponding to $C_8H_7O_2$. The ^{1}H-, ^{13}C-NMR and MS spectra of **3** suggested the presence of anisic acid. The alkaline hydrolysis of **3** gave **3a** and the signal at δ 4.95 in **3** was shifted upfield (Δ −1.47 ppm; presumably located at C-1 or C-9, by comparison with the ^{1}H-NMR spectrum of **2**). Another 1H signal (δ 3.63) was assigned the proton on carbon bearing a hydroxyl group (C-6).

The presence of an anisoyl ester moiety at C-9 was revealed by a ^{1}H-NMR difference spin decoupling experiment (Fig. 1), and the relative stereochemistry was elucidated on the basis of NOE difference experiments; irradiation at 10β-CH_3 (δ 0.95) caused an increase of the signal intensity of the 2β-, 6β-, 8β-, 2′- and 6′-protons. Irradiation at the 6β- and 9α-protons (δ 3.70, 4.95) caused an increase of the signal intensity of the exomethylene and 10β-CH_3 protons, and 1α-, 7α- and 5α-protons, respectively. Consequently, the structure of **3** was established as 6α-hydroxyeudesm-4(15)-ene-9β-*O*-anisate.

Compound **4** was obtained as colorless needles of mp 162—163 °C, and its formula, $C_{15}H_{22}O_3$, was determined by HR-MS.

The IR spectrum showed hydroxyl (3424 cm^{-1}), carbonyl (1682 cm^{-1}) and double bond (1647 cm^{-1}) absorptions. The ^{1}H-NMR spectrum of **4** exhibited two singlet methyl signals at δ 1.04 (3H, s) and 1.60 (3H, s), two methine protons at δ 3.02 (1H, br s) and 3.39 (1H, dd, J=3.9, 12.1 Hz), and two olefinic protons at δ 5.60 and 6.18 (each 1H, s). Acetylation of **4** by the conventional method gave a monoacetate **4a**. The ^{1}H-NMR spectrum of **4a** exhibited an acetylmethyl signal at δ 2.02 (3H, s). The ^{13}C-NMR spectrum showed the presence of one carboxylic acid (δ 170.90; carboxylic carbonyl).

Methylation of **4** afforded a methylester **4b**, whose ^{13}C-NMR carboxylic carbonyl carbon signal was shifted upfield to δ 168.05 (Δ −2.85 ppm).

A two dimensional (2D) homonuclear correlation experiment[7] enabled us to perform an

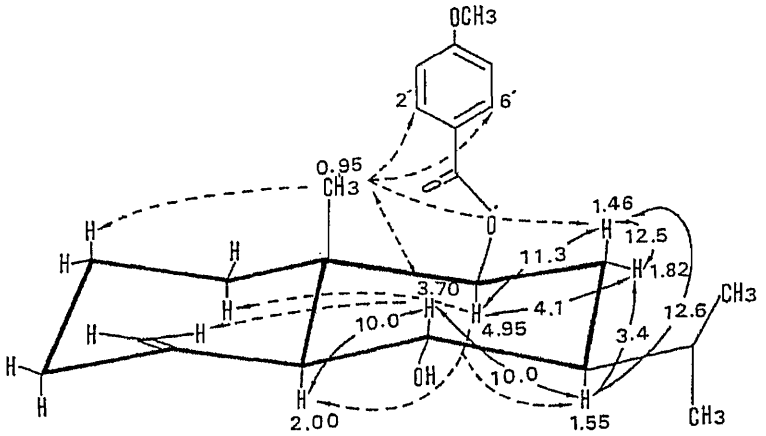


Fig. 1. Partial ^{1}H-NMR Data of **3**

TABLE III. ^{13}C-NMR Chemical Shifts of **4**, **4a** and **4b**

Carbon No.	**4**[a]	**4a**[a]	**4b**[b]
1	78.02	81.21	77.29
2	27.69	24.73	24.06
3	32.85	32.29	31.80
4	133.35	133.30	131.80
5	125.44	125.30	124.65
6	29.54	29.37	28.54
7	35.90	36.64	34.52
8	25.12	25.03	26.91
9	33.26	33.36	32.12
10	40.73	39.68	39.51
11	145.98	148.69	144.02
12	170.90	172.89	168.05
13	125.00	124.54	124.53
14	18.22	19.28	19.02
15	19.23	19.28	17.61
CH_3CO-		21.16	
CH_3CO-		172.89	
-$COOCH_3$			51.76

a) In CD_3OD solution. *b*) In $CDCl_3$ (TMS as an internal standard).

unambiguous assignment.

Figure 2 shows the contour plot of the correlated spectrum of **4**.

The signal of a methine proton at δ 3.39 (H_a) was correlated to the methylene proton signals at δ 1.69 (H_b) and 1.75 (H_c), the signals at δ 1.69 (H_b) and 1.75 (H_c) being correlated to the methylene proton signals at δ 2.00 (H_d) and 2.13 (H_e). Likewise, the signal due to a methine proton at δ 3.02 (H_h) was correlated to the exomethylene proton signals at δ 5.60 (H_m) and 6.18 (H_n), and the signals of two methylene groups at δ 1.63 (H_i) and 1.91 (H_j), and 2.31 (H_g) and 2.68 (H_f). Furthermore, the methylene group signals at δ 1.63 (H_i) and 1.91 (H_j) were correlated to the methylene signals at δ 1.30 (H_k) and 1.69 (H_l). W-Coupling between δ 1.63 (H_i) and 2.68 (H_f) was also observed. Aassignments were made by means of a 2D-heteronuclear correlation experiment,[8] which revealed the directly bonded proton-carbon

TABLE IV. ^{1}H-NMR Chemical Shifts and Coupling Constants of **4**, **4a** and **4b**

Proton No.		**4**[a]	**4a**[a]	**4b**[b]
1	α H_a	3.39 (1H, dd, J=3.9, 12.1 Hz)	4.67 (1H, dd, J=4.0, 12.2 Hz)	3.46 (1H, dd, J=4.8, 11.3 Hz)
2	H_b	1.69 (1H, m)		
	H_c	1.75 (1H, m)		
3	H_d	2.00 (1H, m)		2.02 (1H, m)
	H_e	2.13 (1H, m)	2.18 (1H, m)	2.15 (1H, m)
6	α H_f	2.68 (1H, dd, J=1.7, 15.1 Hz)		2.63 (1H, dd, J=1.7, 15.1 Hz)
	β H_g	2.31 (1H, brd, J=15.1 Hz)	2.73 (1H, brd, J=15.3 Hz)	
7	α H_h	3.02 (1H, m)	3.06 (1H, m)	3.07 (1H, m)
8	α H_i	1.63 (1H, m)		1.62 (1H, m)
	β H_j	1.91 (1H, m)		1.92 (1H, ddd, J=3.4, 13.8, 13.9 Hz)
9	α H_k	1.30 (1H, dd, J=3.4, 13.5 Hz)		1.29 (1H, dd, J=3.4, 13.5 Hz)
	β H_l	1.69 (1H, m)		
13	H_m	5.60 (1H, brs)	5.26 (1H, brs)	5.58 (1H, brs)
	H_n	6.18 (1H, brs)	5.84 (1H, brs)	6.17 (1H, brs)
14		1.04 (3H, s)	1.14 (3H, s)	1.05 (3H, s)
15		1.60 (3H, s)	1.64 (3H, s)	1.59 (3H, s)
$\underline{CH_3}CO$–			2.02 (3H, s)	
–COO$\underline{CH_3}$				3.73 (3H, s)

a) In CD_3OD solution. *b*) In $CDCl_3$ solution (TMS as an internal standard).

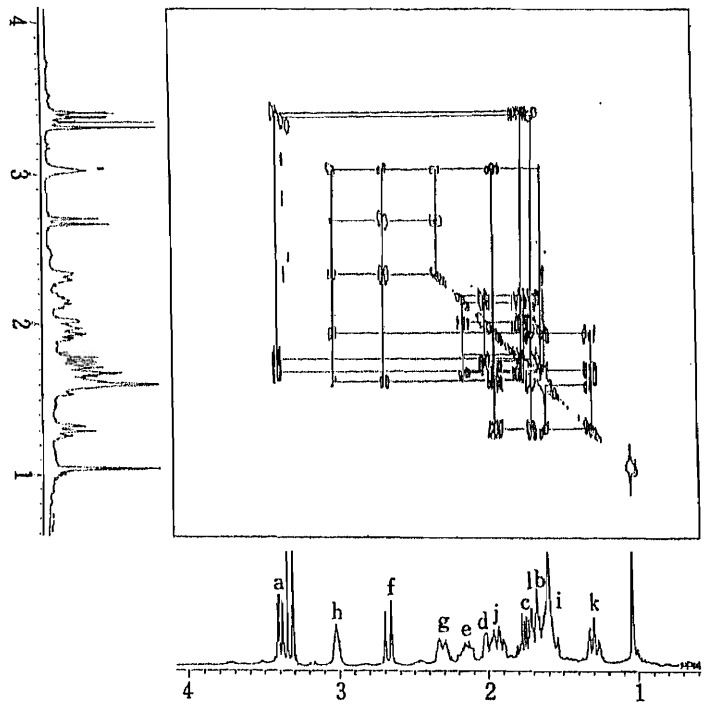


Fig. 2. Contour Plot of the ^{1}H–^{1}H Shift-Correlated Spectrum of **4** in CD_3OD

A 256×2k data matrix was used. To improve the spectral resolution, these data were multiplied in both the f1 and f2 directions with a sine bell function. Fourier transformation was performed with zero filling in the f1 direction. The olefinic protons at δ 5.60 (H_m) and 6.18 (H_n) are outside the spectrum.

relationships. The assignment of the carbon signals is summarized in Table III. The ^{13}C-NMR spectrum of **4a** showed that the signals of C-2 and C-10 were shifted upfield to δ 24.73 (Δ −2.96 ppm) and 39.68 (Δ −1.05 ppm) while that of C-1 was shifted downfield to δ 81.21 (Δ +3.19 ppm).

On the basis of these data, **4** was presumed to be 1β-hydroxyeudesma-4,11(13)-dien-12-oic acid. In order to confirm this structure, a 2D long-range proton–carbon correlation experiment was performed. Three-bond proton–carbon ($^3J_{C-H}$) couplings were detected

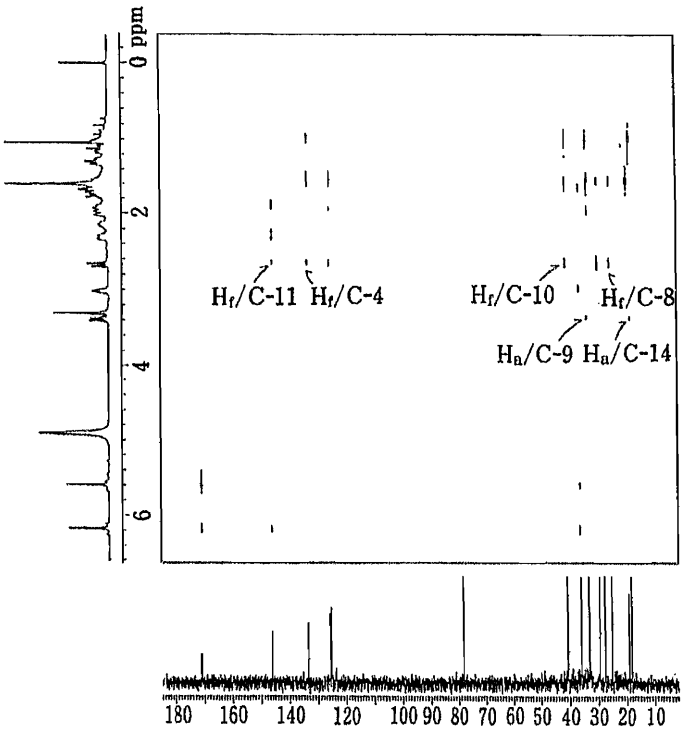


Fig. 3. Contour Plot of a Long-Range ^1H–^{13}C Shift Correlation Spectrum of **4** in CD_3OD

A 256 × 2k data matrix was used. To improve the spectral resolution, the data were multiplied in both the f1 and f2 directions with an exponential function.

between H_f (δ 2.68) and C-10 (δ 40.73), C-4 (δ 133.35) and C-11 (δ 145.98). $^3J_{C-H}$ couplings were detected between H_a (δ 3.39) and C-14 (δ 18.22) and C-9 (δ 33.26). In addition, $^3J_{C-H}$ coupling was observed between H_f (δ 2.68) and C-8 (δ 25.12).

The relative stereochemistry of **4** was deduced from a study of the NOE difference spectrum and coupling constants of each proton. Irradiation at 10β-CH_3 (δ 1.04) and 1α-H (δ 3.39) increased the signal intensity of 8β-H (δ 1.91) and 9α-H (δ 1.30), respectively. Irradiation at 6-H (δ 2.63) increased the signal intensity of 15-CH_3 (δ 1.60). The signal at δ 2.68 was assigned to the 6α-proton. The vicinal coupling between 6α-H and 7-H was so small that 7-H was concluded to be an α-proton. These observations led us to conclude the structure to be **4**.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded on Hitachi 260-30 and Perkin Elmer 1710 FT-IR instruments. ^1H-NMR, ^{13}C-NMR and mass spectra were taken on JEOL GX-400 (at 400 MHz), JEOL GX-400 (at 100 MHz) and JEOL DX-300 spectrometers, respectively. Optical rotations were measured on a JEOL DIP-4 digital polarimeter. Chromatographic purification was carried out on alumina (Wako, 300 mesh) and silica gel (Wako, C-200: Fuji Davison Chemical Co.). Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck, Kieselgel 60 F_{254}).

Extraction and Fractionation——The aerial parts of *Ambrosia elatior* (73.5 kg) were collected in August, 1980, at Funabashi, Chiba Prefecture, and extracted with MeOH at 80 °C for 24 h. The extract was partitioned between ether and water. The total ether solubles were shaken with 5% $NaHCO_3$, 5% Na_2CO_3 and 5% NaOH, successively. The neutral fraction (202 g) was chromatographed on silica gel (200 g, upper layer) and alumina (1.5 kg, bottom layer) with benzene–$CHCl_3$ as an eluent to give fractions 1 (40 g) and 2 (2.1 g). Likewise, the $NaHCO_3$-soluble fraction (92 g) was chromatographed on silica gel with $CHCl_3$ as an eluent to give fractions 3 (15.2 g) and 4 (7.3 g).

Isolation of Damsinic Acid (1)——Fraction 3 (15.2 g) eluted with $CHCl_3$ was repeatedly chromatographed on silica gel with $CHCl_3$. The product was recrystallized from benzene–$CHCl_3$ to give compound **1** (31.9 mg) as colorless needles, mp 120—121 °C. $[\alpha]_D^{15.5}$ +29.6° (c=0.026, $CHCl_3$) (lit.,[5] mp 112—113 °C, $[\alpha]_D^{25}$ +94° (c=0.057, EtOH)). MS *m/z*: 250 (M^+, 24%), 232 (15), 137 (21), 43 (11). HR-MS: Calcd for $C_{15}H_{22}O_3$: 250.1569. Found: 250.1569. IR ν_{max}^{KBr} cm^{-1}: 3400, 2873, 1724, 1704, 1251, 1231, 1141, 942. ^1H-NMR (C_5D_5N) δ: 0.95 (3H, d, J=7.3 Hz, H-14), 1.00 (3H, s, H-15), 1.54 (1H, m, H-2), 1.61—1.67 (2H, m, H-9), 1.75 (1H, H-2), 1.77 (1H, H-8), 1.85 (1H, dd, J=12.2, 14.6 Hz, H-6), 1.90 (1H, m, H-10), 1.98 (1H, m, H-8), 2.08 (1H, m, H-3), 2.15 (1H, m, H-1), 2.38 (1H, m, H-3), 2.54 (1H, dd,

J=3.7, 14.6 Hz, H-6), 3.08 (1H, m, H-7), 5.62 (1H, br s, H-13), 6.43 (1H, br s, H-13). ^{13}C-NMR (C_5D_5N) δ: 17.43 (C-14), 21.31 (C-15), 22.72 (C-2), 31.47 (C-8), 34.24 (C-9), 35.12 (C-10), 35.96 (C-3), 37.05 (C-7), 38.75 (C-6), 46.18 (C-1), 51.24 (C-5), 121.51 (C-13), 149.44 (C-11), 169.70 (C-12), 220.31 (C-4).

Isolation of 1β,6α-Dihydroxyeudesm-4(15)-ene (2)——Fraction 1 (40 g) eluted with benzene–$CHCl_3$ was repeatedly chromatographed on silica gel with benzene–$CHCl_3$ (9 : 1). The product was recrystallized from acetone to give compound **2** as colorless needles, mp 124—125 °C. $[\alpha]_D^{15.5}$ +42.2° (c=0.028, $CHCl_3$). MS m/z: 238 (M^+, 4%), 220 (M^+-H_2O, 51), 202 (19), 190 (M^+-2H_2O, 11), 134 (25), 125 (21), 122 (37), 120 (36), 43 (53), 28 (100). HR-MS m/z: Calcd for $C_{15}H_{26}O_2$: 238.1933. Found: 238.1918. IR ν_{max}^{KBr} cm^{-1}: 3260, 2925, 2850, 1618, 1460, 1440, 1375, 1330, 1285, 1255, 1170, 1050, 1000. ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, H-14), 0.86 (3H, d, J=7.0 Hz, H-12), 0.95 (3H, d, J=7.0 Hz, H-13), 1.15 (1H, H-9), 1.22 (1H, H-9), 1.29 (1H, H-7α), 1.53 (1H, H-8), 1.53 (1H, H-2β), 1.73 (1H, br d, H-5α), 1.84 (1H, dddd, J=2.2, 4.8, 5.2, 12.0 Hz, H-2α), 1.91 (1H, H-8), 2.06 (1H, ddd, J=5.2, 13.0, 13.4 Hz, H-3β), 2.23 (1H, sept d, J=2.0, 7.0 Hz, H-11), 2.32 (1H, ddd, J=2.2, 2.5, 13.0 Hz, H-3β), 3.41 (1H, dd, J=4.8, 11.8 Hz, H-1α), 3.70 (1H, dd, J=10.0, 10.0 Hz, H-6β), 4.73 (1H, br s, H-15), 5.01 (1H, br s, H-15). ^{13}C-NMR: see Table I.

Acetylation of 2——**2** (16 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (1 ml) at room temperature overnight to afford a mixture of products, which was separated by silica gel chromatography to afford **2a** (10.2 mg) and **2b** (3 mg).

2a: Pale yellow oil. $[\alpha]_D^{23.5}$ +20.7° (c=0.010, $CHCl_3$). MS m/z: 280 (M^+, 1%), 262 (M^+–AcOH, 21), 202 (M^+–2AcOH, 46), 159 (100), 43 (93). IR ν_{max}^{KBr} cm^{-1}: 3450, 2950, 1725, 1640, 1360, 1230. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, d, J=7.0 Hz, H-12), 0.88 (3H, s, H-14), 0.94 (3H, d, J=7.0 Hz, H-13), 1.30 (1H, H-7α), 1.59 (1H, H-2β), 1.85 (1H, d, J=10.0 Hz, H-5α), 1.89 (1H, dddd, J=2.2, 4.8, 5.2, 12.0 Hz, H-2α), 2.03 (3H, s, $\underline{CH_3}CO$-1), 2.12 (1H, ddd, J=5.2, 13.0, 13.4 Hz, H-3α), 2.24 (1H, sept d, J=2.0, 7.0 Hz, H-11), 2.35 (1H, ddd, J=2.2, 2.5, 13.0 Hz, H-3β), 3.71 (1H, dd, J=10.0, 10.0 Hz, H-6β), 4.67 (1H, dd, J=4.8, 11.8 Hz, H-1α), 4.77 (1H, br s, H-15), 5.05 (1H, br s, H-15). ^{13}C-NMR ($CDCl_3$) δ: 12.67 (C-14), 16.24 (C-12), 18.11 (C-8), 21.05 (C-13), 21.16 ($\underline{CH_3}CO$-1), 26.05 (C-11), 28.55 (C-2), 34.84 (C-9), 36.10 (C-3), 40.80 (C-10), 49.36 (C-7), 55.99 (C-5), 66.89 (C-6), 80.22 (C-1), 108.30 (C-15), 145.70 (C-4), 170.67 ($CH_3\underline{CO}$-1).

2b: Colorless needles, mp 91—93 °C. $[\alpha]_D^{23.5}$ −19.5° (c=0.017, $CHCl_3$). MS m/z: 322 (M^+, 1%), 262 (M^+–AcOH, 21), 202 (M^+–2AcOH, 46), 159 (100), 43 (93). IR ν_{max}^{KBr} cm^{-1}: 2950, 1725, 1640, 1360. ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, s, H-14), 0.86 (3H, d, J=7.0 Hz, H-12), 0.91 (3H, d, J=7.0 Hz, H-13), 1.59 (1H, m, H-2β), 1.88 (1H, m, H-2α), 1.99 (3H, s, $\underline{CH_3}CO$-6), 2.04 (3H, s, $\underline{CH_3}CO$-1), 4.60 (1H, br s, H-15), 4.82 (1H, br s, H-15), 5.08 (1H, dd, J=10.0, 10.0 Hz, H-6α). ^{13}C-NMR ($CDCl_3$) δ: 12.07 (C-14), 16.01 (C-12), 17.91 (C-8), 21.14 (C-13), 21.19 ($\underline{CH_3}CO$-1), 21.31 ($\underline{CH_3}CO$-6), 26.14 (C-11), 28.72 (C-2), 34.80 (C-9), 35.84 (C-3), 41.26 (C-10), 48.92 (C-7), 53.61 (C-5), 70.58 (C-6), 80.30 (C-1), 108.26 (C-15), 144.03 (C-4), 170.64 ($CH_3\underline{CO}$-1), 171.08 ($CH_3\underline{CO}$-6).

Isolation of 6α-Hydroxyeudesm-4(15)-ene-9β-*O*-anisate (3)——Fraction 2 (2.1 g), which was eluted from benzene–$CHCl_3$ (1 : 1) to $CHCl_3$, was repeatedly chromatographed on silica gel with benzene–$CHCl_3$ (9 : 1). The product was recrystallized from benzene–$CHCl_3$ to give compound **3** (20 mg) as colorless needles, mp 191.0—192.0 °C. $[\alpha]_D^{15.5}$ +21.1° (c=0.025, $CHCl_3$). MS m/z: 372 (M^+, 4%), 220 (12), 202 (29), 177 (11), 159 (27), 152 (28), 136 (15), 135 (100), 107 (16), 93 (12), 77 (17), 43 (11), 41 (12). HR-MS m/z: Calcd for $C_{23}H_{32}O_4$: 372.2300. Found: 372.2404. IR ν_{max}^{KBr} cm^{-1}: 3475, 2950, 1710, 1610, 1510, 1455, 1320, 1280, 1255, 1170, 1120, 1030, 970, 850, 770. ^{1}H-NMR: see Table II. ^{13}C-NMR: see Table I.

Hydrolysis of 3——A solution of **3** (3 mg) in MeOH (1 ml) was allowed to react with 10% KOH (0.5 ml) at 80 °C for 4 h, then H_2O (5 ml) was added. The solution was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O and evaporated to give a syrup, which was subjected to preparative TLC with $CHCl_3$–MeOH (100 : 1) to afford **3a** as a gum. The aqueous layer was acidified with 5% HCl. The solution was extracted with $CHCl_3$, and the $CHCl_3$ layer was evaporated to give *p*-methoxybenzoic acid which was identified by TLC comparison with an authentic sample (Kieselgel 60 F_{254}, $CHCl_3$–MeOH (10 : 1), Rf: 0.51). **3a**: A gum. MS m/z: 238 (M^+, 46%), 220 (M^+-H_2O, 60), 202 (M^+-2H_2O, 26), 177 (56), 159 (43), 95 (100), 43 (81). ^{1}H-NMR: see Table II. ^{13}C-NMR: see Table I.

Isolation of 1β-Hydroxyeudesma-4,11(13)-dien-12-oic Acid (4)——Fraction 4 (7.3 g), which was eluted with $CHCl_3$ (100%), was repeatedly chromatographed on a Sephadex LH-20 and silica gel prepacked column to give **4** (45.2 mg) as colorless needles, mp 162—163 °C. $[\alpha]_D^{20}$ −21.8° (c=0.10, EtOH). MS m/z: 250 (M^+, 25%), 232 (M^+-H_2O, 47), 206 (44), 176 (54), 91 (100), 77 (70). HR-MS m/z: Calcd for $C_{15}H_{22}O_3$: 250.15687. Found: 250.15586. IR ν_{max}^{KBr} cm^{-1}: 3424, 1682, 1647, 1621, 1227. ^{1}H-NMR: see Table IV. ^{13}C-NMR: see Table III.

Methyl Ester of 4——MeOH (100 ml) containing 5% dry HCl gas was added to a solution of the crude product from fraction 4 (10 g) in MeOH (50 ml), and the mixture was allowed to stand for 24 h at room temperature. The reaction mixture was worked up as usual and the product was purified by silica gel chromatography and preparative TLC to afford the methyl ester (**4a**, 45.6 mg) as a yellow oil. IR ν_{max}^{KBr} cm^{-1}: 3450, 1771, 1622, 1456, 1259. ^{1}H-NMR: see Table IV. ^{13}C-NMR: see Table IV.

Acetylation of 4——**4** (2 mg) was dissolved in pyridine (0.5 ml) and Ac_2O (0.4 ml), and the reaction mixture was left overnight at room temperature. The reagents were evaporated off *in vacuo* and the residue was purified by preparative TLC to give a monoacetate **4b** (1 mg), gum. MS m/z: 292 (M^+, 1%), 232 (M^+–AcOH, 20), 176 (33), 44 (100), 43 (52). ^{1}H-NMR: see Table IV. ^{13}C-NMR: see Table III.

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Pyridonecarboxylic Acids as Antibacterial Agents. VIII.[1] An Alternative Synthesis of Enoxacin *via* Fluoronicotinic Acid Derivatives

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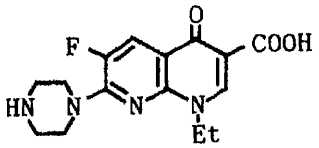
An alternative synthesis of enoxacin, a 1,8-naphthyridine antibacterial agent, was developed. The present method involves 1,8-naphthyridine ring construction by the Dieckmann type cyclization of ethyl 5-fluoronicotinate having a 2-ethoxycarbonylethylamino moiety at C-2. This nicotinate was prepared in 7 steps from ethyl fluoroacetate *via* ethyl 2,6-dichloro-5-fluoronicotinate.

Keywords——ethyl fluoroacetate; ethyl 5-fluoronicotinate; 1,8-naphthyridine; Dieckmann cyclization; antibacterial agent; enoxacin

Enoxacin (**1**),[2a] a pyridonecarboxylic acid antibacterial agent, has recently been introduced as a chemotherapeutic agent. Our previous methods[2] for the synthesis of enoxacin have involved the introduction of a fluorine atom into a pyridine or 1,8-naphthyridine ring by means of the Balz–Schiemann reaction, which is a key step in the sequence of reactions.

Santilli *et al.*[3] have reported a construction of the 1,8-naphthyridine skeleton of nalidixic acid by the Dieckmann-type cyclization of a nicotinate derivative. Efforts were first focussed on the preparation of ethyl 2,6-dichloro-5-fluoronicotinate (**10**) which would be an intermediate for enoxacin in the route involving the Dieckmann-type cyclization.



1 (enoxacin)

Chart 1

Hirota *et al.*[4] have reported the ring transformation of 5-fluoro-1,3-dimethyluracil to 5-fluoro-2,6-dihydroxynicotinamide (**5**), which could be converted to the requisite compound **10**. Their method, however, has the disadvantages that the yield is unsatisfactory (38%) and 5-fluorouracil is very expensive. Therefore, commercially available ethyl fluoroacetate (**2**) was used as a starting material in the present study.

Reaction of **2** with ethyl formate in the presence of sodium hydride in ether, followed by treatment with trimethylsilyl chloride, afforded ethyl 2-fluoro-3-(trimethylsiloxy)acrylate (**3**). When **3** was allowed to react with malonamide in the presence of sodium ethoxide in boiling ethanol, **5** was obtained in 65% yield. Compound **5** was identical with an authentic specimen prepared by the reported method.[4a] One-pot preparation of **5** from **2** was accomplished in the following manner; **2** was treated with ethyl formate and sodium ethoxide to give the sodium salt of ethyl formylfluoroacetate (**4**), which, without isolation, was allowed to react with malonamide in boiling ethanol to yield **5** in 63% yield. According to the same procedure, 5-

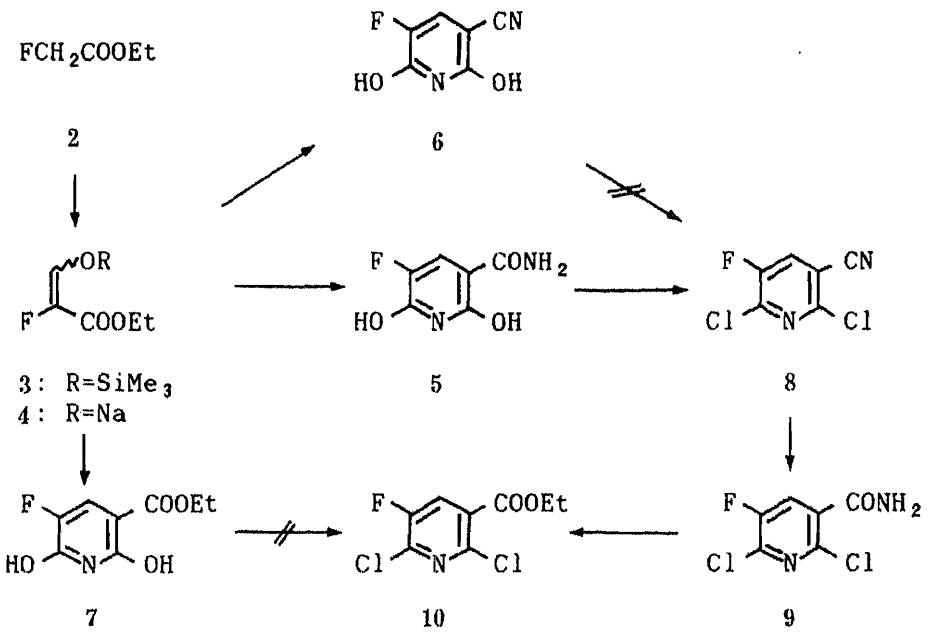


Chart 2

fluoro-2,6-dihydroxynicotinonitrile (**6**) and the corresponding nicotinate analogue **7** were prepared from cyanoacetamide and ethoxycarbonylacetamide in 57% and 75% yields, respectively. Treatment of **5** with phosphorus pentachloride resulted in chlorination with concomitant dehydration to give 2,6-dichloro-5-fluoronicotinonitrile (**8**). However, attempted conversions of **6** to **8** and of **7** to **10** both failed under the same conditions. Hence, the nitrile **8** was hydrolyzed to the amide **9**, which was then converted to the intermediate **10** by treatment with boron trifluoride-etherate in ethanol.

The chloro group at C-2 in **8** and **10** was replaced by an appropriate amine. The displacement reaction of **10** with *N*-acetylpiperazine proceeded regioselectively in a solvent such as acetonitrile, ethanol or toluene to give the corresponding 6-substituted compound **11a** in good yield. In order to confirm the structure of **11a**, dehalogenation of **11a** to **12a** was carried out by catalytic hydrogenolysis. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **12a** shows a double doublet at δ 7.77 ($J_{F,H}=7$ Hz, $J_{H,H}=2$ Hz) and a triplet at δ 8.66 ($J_{F,H}$ and $J_{H,H}=2$ Hz) which are assignable to the C-4 proton and the C-2 proton, respectively. This observation permits assignment of the site of the displacement with *N*-acetylpiperazine as position 6 in **10**. Similarly, the reaction of **8** with *N*-acetylpiperazine afforded preferentially the 6-substituted compound **11b**, as expected. The structure of **11b** was confirmed by the ^{1}H-NMR spectrum of **12b**, which was prepared from **11b** by hydrogenolysis.

When **11a** was heated at 120—130 °C with ethyl 3-(ethylamino)propionate in dimethylformamide (DMF) in the presence of sodium bicarbonate, the desired diester **13** was produced in 42% yield together with a small amount of the 2-ethylamino compound **14a**. The formation of **14a** was owing probably to the reaction of **11a** with ethylamine which would arise from the decomposition of ethyl 3-(ethylamino)propionate during the reaction. This was supported by the fact that the formation of **14a** was not observed when **13** was treated under the same conditions. On the reaction of **11b** with ethyl 3-(ethylamino)propionate under the same conditions, **11b** was recovered unchanged. In the reaction of **11b**, the use of potassium carbonate instead of sodium bicarbonate at a temperature above 150 °C gave the undesired compound **14b** as a main product. Authentic specimens of **14a** and **14b** were prepared by the reaction of **11a** and **11b**, respectively, with ethylamine in a sealed tube.

On treatment with sodium hydride or potassium *tert*-butoxide in toluene, the diester **13**

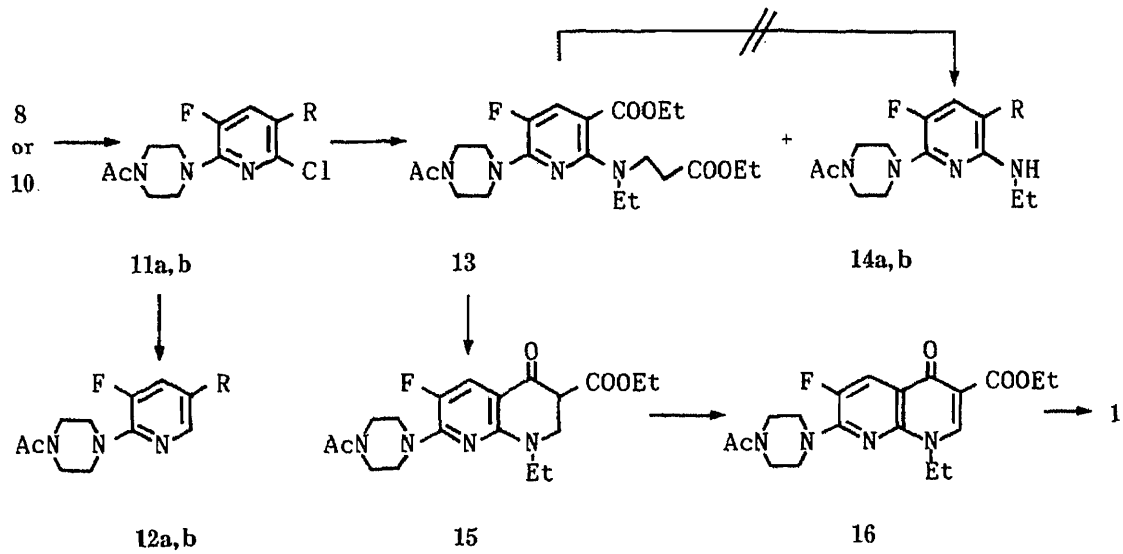


Chart 3

underwent Dieckmann-type cyclization to give the tetrahydronaphthyridine derivative **15** in good yield. The ^{1}H-NMR spectrum of **15** in deuteriochloroform shows that **15** exists mainly in keto form. Oxidation of **15** with an equivalent amount of chloranil and pyridine in chloroform gave the precursor **16** for enoxacin in 88% yield. Without the use of pyridine, the yield of **16** was less than 50%. Hydrolysis of **16** to enoxacin has already been described in our previous paper.[2a)]

Experimental

Melting points were determined on a Yanagimoto micromelting point apparatus. Boiling points and melting points are uncorrected. Infrared (IR) spectra were recorded on a Jasco A-102 or a Hitachi 215 spectrometer. ^{1}H-NMR spectra were taken at 60 MHz with a Varian EM-360A, at 80 MHz with a Varian FT-80A or at 100 MHz with a Varian HA-100D spectrometer. Chemical shifts are expressed in δ (ppm) values with tetramethylsilane as an internal standard. Mass spectra (MS) were recorded on a JEOL JMS D-300. Physical and analytical data for nicotinic acid derivatives are listed in Tables I and II, respectively.

Ethyl 2-Fluoro-3-(trimethylsiloxy)acrylate (3)——Ethyl fluoroacetate (2) (106 g, 1.0 mol) was added dropwise to a stirred mixture of ethyl formate (74 g, 1.0 mol), NaH (60% dispersion in mineral oil, 85 g, 2.1 mol) and dry Et_2O (650 ml) over a period of 2 h under ice-cooling. The mixture was vigorously stirred for 2 h at room temperature. After addition of trimethylsilyl chloride (278 ml) over a period of 1.5 h under ice-cooling, the resulting mixture was stirred for an additional 2 h at room temperature. The insoluble material was removed by filtration. The filtrate was concentrated to dryness and the resulting crude product was purified by distillation to give **3** (142 g, 69%); bp 80—86 °C (8 mmHg). *Anal.* Calcd for $C_8H_5FO_3Si$: C, 46.58; H, 7.33; F, 9.21. Found: C, 47.00; H, 7.47; F, 9.09. IR (neat) cm^{-1}: 1730, 1670. NMR (60 MHz, $CDCl_3$): 1.00 (3H, t, J=7 Hz), 3.98 (2H, q, J=7 Hz), 6.76 (1H, d, J=19 Hz).

5-Fluoro-2,6-dihydroxynicotinamide (5)——Method A: A mixture of **4** (41.2 g, 0.20 mol), malonamide (41.0 g, 0.40 mol), EtONa (27.2 g, 0.40 mol) and EtOH (420 ml) was heated to reflux for 20 min with vigorous stirring. After addition of water (200 ml), the mixture was acidified with conc. HCl (60 ml). The resulting solid was collected by filtration, and washed successively with water and EtOH to give the crude product, **5**, (22.2 g, 65%), which was dissolved in aq. NH_3. The solution was treated with charcoal and acidified with conc. HCl to give **5**, mp >300 °C (lit.[4a)] mp >300 °C).

Method B: Under ice-cooling, EtONa, (10.0 g, 147 mmol) was gradually added to a stirred mixture of ethyl fluoroacetate (10.6 g, 103 mmol) and ethyl formate (9.2 ml, 114 mmol). The mixture was stirred for 3 h at room temperature. Then malonamide (11.8 g, 115 mmol) and EtOH (120 ml) were added. The mixture was heated to reflux for 15 min with vigorous stirring and diluted with water. After addition of conc. HCl (20 ml), the resulting solid was collected by filtration, and washed successively with water and EtOH to give **5** (11.2 g, 63%).

5-Fluoro-2,6-dihydroxynicotinonitrile (6)——According to method B for the preparation of **5**, a mixture of ethyl

TABLE I. Physical Data for Nicotinic Acid Derivatives

Compd.	mp (°C) (Recrystn. solvent)	IR (KBr) cm^{-1}	^{1}H-NMR (MHz, solvent) δ (ppm)
6	235—245 (H_2O)	2220, 1630	(60, DMSO-d_6) 7.58 (1H, d, J=8 Hz)
7	201—204 (AcOEt)	1620	(60, $CDCl_3$) 1.37 (3H, t, J=7 Hz), 4.36 (2H, q, J=7 Hz), 7.51 (1H, d, J=9 Hz)
8	91—93 (Et_2O–hexane)	2220	(60, $CDCl_3$) 7.76 (1H, d, J=6.5 Hz)
9	163—164 (Et_2O)	3380, 3170, 1655	(80, DMSO-d_6) 7.5—8.4 (2H, br), 8.20 (1H, d, J=8 Hz)
10	95—97[a] [2—3 mmHg]	1735[b]	(60, $CDCl_3$) 1.43 (3H, t, J=7 Hz), 4.44 (2H, q, J=7 Hz), 7.98 (1H, d, J=9 Hz)
11a	69—70 (Et_2O–hexane)	1705, 1685, 1625	(60, $CDCl_3$) 1.37 (3H, t, J=7 Hz), 2.12 (3H, s), 3.4—4.0 (8H, m), 4.32 (2H, q, J=7 Hz), 7.80 (1H, d, J=13 Hz)
11b	166—167 (EtOH)	2220, 1640	(60, $CDCl_3$) 2.12 (3H, s), 3.4—4.0 (8H, m), 7.47 (1H, d, J=12 Hz)
12a	77—78 (Et_2O–hexane)	1705, 1635	(80, $CDCl_3$) 1.38 (3H, t, J=7 Hz), 2.15 (3H, s), 3.4—3.9 (8H, m), 4.35 (2H, q, J=7 Hz), 7.77 (1H, dd, J=11, 2 Hz), 8.60 (1H, t, J=2 Hz)
12b	138—139 (AcOEt–hexane)	2220, 1645	(80, $CDCl_3$) 2.14 (3H, s), 3.4—3.9 (8H, m), 7.42 (1H, dd, J=13, 2 Hz), 8.24 (1H, t, J=2 Hz)
13	—	1720, 1710, 1640[b]	(100, $CDCl_3$) 1.14, 1.24 and 1.33 (each 3H, t, J=7 Hz), 2.12 (3H, s), 2.64 (2H, t, J=7 Hz), 3.31, 4.12 and 4.29 (each 2H, q, J=7 Hz), 3.4—3.9 (10H, m), 7.66 (1H, d, J=13 Hz)
14a	91—92 (Et_2O–hexane)	3340, 1670, 1630	(60, $CDCl_3$) 1.22 and 1.34 (each 3H, t, J=7 Hz), 2.12 (3H, s), 3.4—4.0 (8H, m), 3.41 and 4.28 (each 2H, q, J=7 Hz), 7.6—8.1 (1H, br), 7.70 (1H, d, J=14 Hz)
14b	128—129 (AcOEt–hexane)	3340, 2210, 1630	(80, $CDCl_3$) 1.21 (3H, t, J=7 Hz), 2.12 (3H, s), 3.4—3.9 (8H, m), 3.44 (2H, q, J=7 Hz), 4.7—5.0 (1H, br), 7.15 (1H, d, J=13 Hz)

a) bp (°C). b) Neat.

TABLE II. Analytical Data for Nicotinic Acid Derivatives

Compd.	Formula	Calcd (%)					Found (%)				
		C	H	Cl	F	N	C	H	Cl	F	N
6	$C_6H_3FN_2O_2$	46.76	1.96		12.33	18.18	46.94	1.88		12.54	17.96
7	$C_8H_8FNO_4$	47.77	4.01		9.44	6.96	47.78	4.07		9.34	7.13
8	$C_6HCl_2FN_2$	37.76	0.53	37.13	9.95	14.67	37.74	0.38	37.21	10.04	14.82
9	$C_6H_3Cl_2FN_2O$	34.48	1.45	33.93	9.09	13.40	34.80	1.73	33.97	8.90	13.64
10	$C_8H_6Cl_2FNO_2$	40.37	2.54	29.79	7.98	5.88	40.35	2.58	29.57	7.74	5.88
11a	$C_{14}H_{17}ClFN_3O_3$	50.99	5.20	10.75	5.76	12.74	51.07	5.10	10.91	5.48	12.94
11b	$C_{12}H_{12}ClFN_4O$	50.98	4.28	12.54	6.72	19.82	50.81	4.33	12.55	6.59	19.81
12a	$C_{14}H_{18}FN_3O_3$	56.94	6.14		6.43	14.23	56.73	5.92		6.40	14.16
12b	$C_{12}H_{13}FN_4O$	58.06	5.28		7.65	22.57	58.00	5.09		7.42	22.49
14a	$C_{16}H_{23}FN_4O_3$	56.79	6.85		5.61	16.56	56.96	6.87		5.78	16.44
14b	$C_{14}H_{18}FN_5O$	57.72	6.23		6.52	24.04	57.94	5.99		6.51	24.06

fluoroacetate (10.6 g), ethyl formate (9.2 ml) and EtONa (10.0 g) was treated with cyanoacetamide (9.7 g) to give **6** (9.1 g, 57%).

Ethyl 5-Fluoro-2,6-dihydroxynicotinate (7)——According to method B for the preparation of **5**, a mixture of ethyl fluoroacetate (8.5 g), ethyl formate (7.4 ml) and EtONa (8.0 g) was treated with ethoxycarbonylacetamide

(12.1 g) to give **7** (12.5 g, 75%).

2,6-Dichloro-5-fluoronicotinonitrile (8)——A mixture of **5** (7.5 g, 436 mmol) and PCl_5 (30.0 g) was heated until a solution was formed at 140 °C, and stirred for 2.5 h at 130 °C. After removal of the resulting $POCl_3$ under reduced pressure, the residue was poured into ice-water while the temperature was maintained at 30—40 °C. The resulting solid was collected by filtration and washed successively with water and a mixture of water and iso-PrOH (1 : 1) to give **8** (6.4 g, 77%).

2,6-Dichloro-5-fluoronicotinamide (9)——A stirred mixture of **8** (10.0 g, 52.4 mmol) and conc. H_2SO_4 (50 ml) was heated at 60—65 °C for 1 h. The solution was poured into ice-water (200 ml) and extracted with AcOEt. The extract was dried over Na_2SO_4 and concentrated to dryness. After addition of hexane, the resulting solid was collected by filtration to give **9** (9.2 g, 84%).

Ethyl 2,6-Dichloro-5-fluoronicotinate (10)——A mixture of **9** (9.0 g, 43.1 mmol), BF_3–Et_2O (36 ml) and abs. EtOH (90 ml) was heated at 60 °C for 30 min, during which period the Et_2O was removed, and then refluxed for an additional 16 h. After removal of the solvent under reduced pressure, ice-water (150 ml) was added to the residue. The mixture was extracted with toluene. The extract was dried over Na_2SO_4 and concentrated to dryness. The crude product was purified by distillation to give **10** (8.1 g, 79%).

Ethyl 6-(4-Acetyl-1-piperazinyl)-2-chloro-5-fluoronicotinate (11a)——A mixture of **10** (4.0 g, 16.8 mmol), *N*-acetylpiperazine (3.2 g, 25.2 mmol), triethylamine (3.5 ml) and MeCN (20 ml) was heated to reflux for 1.5 h and then concentrated to dryness under reduced pressure. After addition of dil. HCl, the mixture was extracted with toluene. The extract was dried over Na_2SO_4 and concentrated to dryness. After addition of hexane, the resulting solid was collected by filtration to give **11a** (5.2 g, 94%). When EtOH or toluene was used as a solvent instead of MeCN, the yield of **11a** was 87% or 84%, respectively.

6-(4-Acetyl-1-piperazinyl)-2-chloro-5-fluoronicotinonitrile (11b)——A mixture of **8** (1.0 g, 5.2 mmol), *N*-acetylpiperazine (0.8 g, 6.3 mmol), triethylamine (0.9 ml) and MeCN (20 ml) was stirred for 2 h at room temperature and then concentrated to dryness under reduced pressure. After addition of dil. HCl, the mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and concentrated to dryness. After addition of AcOEt, the resulting solid was collected by filtration to give **11b** (1.2 g, 81%). When EtOH or toluene was used as a solvent instead of MeCN, the yield of **11b** was 95% or 74%, respectively.

Ethyl 6-(4-Acetyl-1-piperazinyl)-5-fluoronicotinate (12a)——In the presence of 5% palladium-on-charcoal (100 mg) and triethylamine (0.5 ml), **11a** (1.0 g, 3.0 mmol) was hydrogenated in EtOH (20 ml) at room temperature until the required volume of hydrogen (*ca.* 70 ml) was absorbed. The catalyst was removed by filtration and then the filtrate was concentrated to dryness. After addition of hexane, the resulting solid was collected by filtration to give **12a** (0.8 g, 90%).

6-(4-Acetyl-1-piperazinyl)-5-fluoronicotinonitrile (12b)——According to the method described for the preparation of **12a**, **11a** (0.5 g, 1.8 mmol) was worked up to give **12b** (0.4 g, 91%).

Ethyl 6-(4-Acetyl-1-piperazinyl)-2-[*N*-ethyl-*N*-(2-ethoxycarbonylethyl)]amino-5-fluoronicotinate (13)——A mixture of **11a** (2.7 g, 8.2 mmol), ethyl 3-ethylaminopropionate (2.4 g, 16.4 mmol), $NaHCO_3$ (1.4 g, 16.4 mmol) and DMF (54 ml) was heated at 120—130 °C for 8.5 h with vigorous stirring. The insoluble material was removed by filtration and the filtrate was concentrated to dryness under reduced pressure. The residue was taken up in water and toluene. The toluene layer was separated and extracted with 10% HCl. The aqueous layer was made alkaline with K_2CO_3 and extracted with toluene. The extract was dried over Na_2SO_4, treated with charcoal, and then concentrated to dryness to give the oil **13** (1.5 g, 42%). MS *m/z*: 438 (M^+) 423, 393, 363, 351, 337, 291.

The toluene layer which had been extracted with 10% HCl was washed with water and dried over Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography on silica gel with $CHCl_3$ as an eluent to give **14a** (0.1 g, 4%).

Ethyl 6-(4-Acetyl-1-piperazinyl)-2-ethylamino-5-fluoronicotinate (14a)——A mixture of **11a** (1.0 g, 3.0 mmol), ethylamine (70% solution in water, 5 ml) and EtOH (15 ml) was heated at 90 °C for 13 h in a sealed tube. The mixture was concentrated to dryness and taken up in water and AcOEt. The organic layer was separated and dried over Na_2SO_4. After removal of the solvent, hexane was added. The resulting solid was collected by filtration and washed with iso-Pr_2O to give **14a** (0.9 g, 88%).

6-(4-Acetyl-1-piperazinyl)-2-ethylamino-5-fluoronicotinonitrile (14b)——i) According to the method described for the preparation of **14a**, **11b** (1.0 g, 3.5 mmol) was worked up to give **14b** (0.8 g, 78%).

ii) A mixture of **11b** (1.0 g, 3.5 mmol), ethyl 3-ethylaminopropionate (1.0 g, 6.9 mmol), K_2CO_3 (0.5 g, 3.6 mmol) and DMF (5 ml) was heated at 140—160 °C for 7.5 h with vigorous stirring, and then concentrated to dryness under reduced pressure. After addition of water, the mixture was extracted with toluene. The extract was washed successively with 10% HCl and water, and dried over Na_2SO_4. After removal of the solvent, the residue was purified by column chromatography on silica gel with $CHCl_3$ as an eluent to give **14b** (0.55 g, 53%).

Ethyl 7-(4-Acetyl-1-piperazinyl)-1-ethyl-6-fluoro-1,2,3,4-tetrahydro-4-oxo-1,8-naphthyridine-3-carboxylate (15)——Potassium *tert*-butoxide (0.4 g, 3.5 mmol) was gradually added to a stirred solution of **13** (1.4 g, 3.2 mmol) in toluene (14 ml). The mixture was stirred for 1 h at room temperature. The resulting precipitate was collected by filtration and dissolved in 1 N AcOH (5 ml). The mixture was extracted with AcOEt, and the extract was dried over

Na_2SO_4. After removal of the solvent, hexane was added. The resulting crystals were collected by filtration to give **15** (1.0 g, 80%), which was recrystallized from a mixture of CH_2Cl_2 and hexane, mp 141—143 °C. *Anal.* Calcd for $C_{19}H_{25}FN_4O_4$: C, 58.15; H, 6.42; F, 4.84; N, 14.28. Found: C, 57.90; H, 6.25; F, 4.59; N, 14.12. IR (KBr) cm^{-1}: 1725, 1635. NMR (100 MHz, $CDCl_3$): 1.17 and 1.27 (each 3H, t, $J=7$ Hz, CH_2CH_3), 2.13 (3H, s, $COCH_3$), 3.3—3.9 (13H, m, C-2, C-3 and piperazinyl H, NCH_2CH_3), 4.22 (2H, q, $J=7$ Hz, OCH_2CH_3), 7.62 (1H, d, $J=13$ Hz, aromatic H).

The use of NaH instead of *tert*-BuOK gave an 80% yield of **15**.

Ethyl 7-(4-Acetyl-1-piperazinyl)-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylate (16)——A mixture of **15** (500 mg, 1.28 mmol), chloranil (315 mg, 1.28 mmol), pyridine (0.1 ml) and $CHCl_3$ (10 ml) was heated to reflux for 30 min. The solution was washed with 1 N NaOH (5 ml) and dried over Na_2SO_4. After removal of the solvent, Et_2O was added. The resulting crystals were collected by filtration to give **16** (440 mg, 88%), which was recrystallized from AcOEt, mp 195—197 °C (lit.[2a] mp 195—197 °C).

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Synthesis of a Bicyclo[3.2.1]octane Analogue of Isocarbacyclin

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A new isocarbacyclin analogue (**1a**), containing a bicyclo[3.2.1]octane ring system, has been synthesized by means of a regioselective rearrangement of the cyclopropyl carbinol (**8**) to the homoallyl bromide (**9**) with hydrobromic acid. Compound **1a** showed very weak inhibitory activity against platelet aggregation.

Keywords——carbacyclin; isocarbacyclin; 3-oxaisocarbacyclin; bicyclo[3.2.1]octane; cyclopropyl'carbinol; homoallyl bromide

Many prostacyclin analogues have been synthesized with the aim of developing therapeutically useful agents.[1] Ring-modified carbacyclin analogues such as a bicyclo[4.3.0]nonane analogue (**2**),[2] a bicyclo[3.2.0]heptane analogue (**3**)[3] and a bicyclo[3.1.0]hexane analogue (**4**)[4] have already been synthesized, but no bicyclo[3.2.1]octane analogue has yet been reported.[5] In the course of our synthetic studies on stable prostacyclin analogues, we wished to synthesize a new bicyclo[3.2.1]octane analogue (**1a**) of 3-oxaisocarbacyclin (**5**), because 3-oxaisocarbacyclin showed fairly potent inhibitory activity against platelet aggregation.[6] We herein describe the synthesis of **1a** by using a regioselective rearrangement of the cyclopropyl carbinol (**8**) to the homoallyl bromide (**9**) with hydrobromic acid as a key step.

The starting tricyclic β-ketoester (**6**) was prepared as described in the previous report.[6] The ketone group in **6** was reduced with sodium borohydride ($NaBH_4$) to afford the unstable

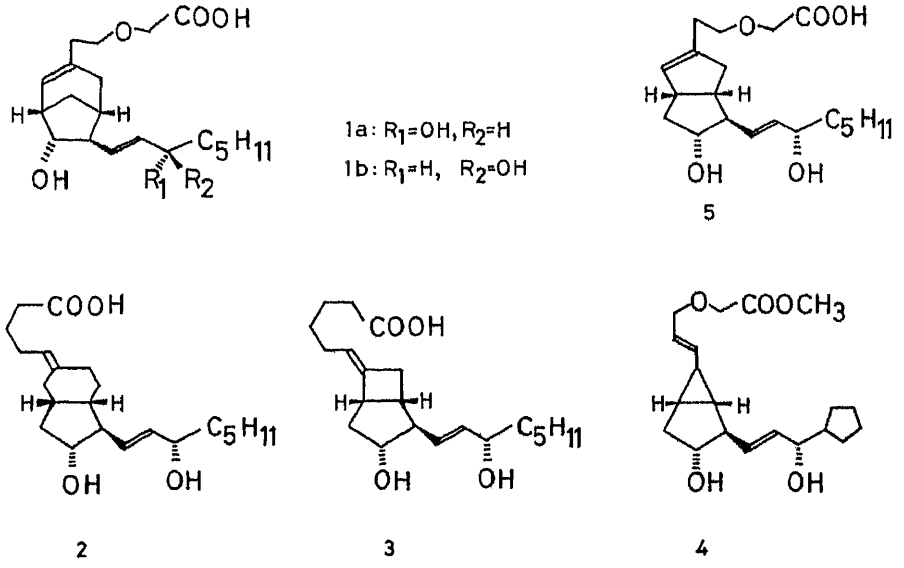


Chart 1

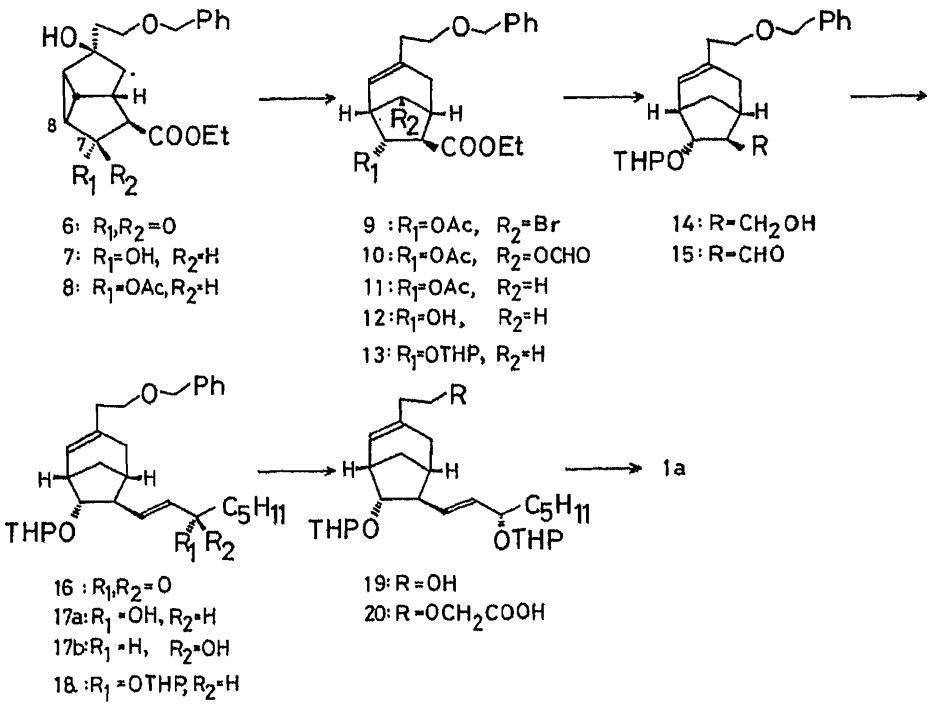
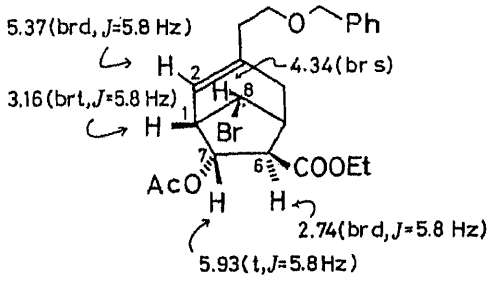


Chart 2

hydroxy ester (**7**), which was acetylated with acetic anhydride and pyridine to give the tricyclic acetate (**8**) in 55% yield from **6**. α-Stereochemistry of the acetoxy group in **8** was assigned on the basis of the steric effects on the $NaBH_4$ reduction and an analysis of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. The ^{1}H-NMR spectrum of **8** shows a signal due to the C_7 proton as a broad doublet with a coupling constant of $J_{7,8}=6$ Hz.

We then carried out the key reaction, the conversion of the cyclopropyl carbinol system in **8** to the homoallylic system in **9**. Treatment of **8** with excess 48% hydrobromic acid in ether under ice-cooling afforded the single bromide (**9**) in 63% yield. The structure of **9** was determined by ^{1}H-NMR assignment including a decoupling experiment, as summarized in Chart 3. Compound **9** still has an acetyl group, suggesting that the cyclopropyl carbinol moiety with the tertiary hydroxy group is, as expected, more reactive than the cyclopropyl carbinol acetate moiety with a secondary acetate function. However, there are two possible routes for the acid rearrangement of the cyclopropyl carbinol group in **8**; one yields a bicyclo[3.2.1]octane skeleton, such as **9**, and the other affords a bicyclo[3.3.0]octane skeleton. The ^{1}H-NMR spectrum of the product exhibits an olefinic proton signal at δ 5.37 as a broad



9

Chart 3

doublet with a coupling constant of $J=5.8$ Hz, which is coupled with the C_1 proton at δ 3.16 observed as a broad triplet ($J=5.8$ Hz). In bicyclo[3.3.0]octane compounds, the olefinic proton is observed as a broad singlet[6] instead of a doublet. The C_1 proton is further coupled with the C_7 proton at δ 5.93[7] observed as a triplet with $J\doteq5.8$ Hz, and the C_7 proton is also coupled with the C_6 proton at δ 2.74 observed as a broad doublet with $J=5.8$ Hz. The proton at C_8 position is observed at δ 4.34 as a broad singlet ($W_{1/2}=5$ Hz), which changes to a sharp singlet ($W_{1/2}=3$ Hz) on irradiation of the C_1 proton at δ 3.16. These ^{1}H-NMR data coupled with molecular model inspection indicate that the bromide (**9**) has a bicyclo[3.2.1]octane skeleton. Similar treatment of **8** with hydrobromic acid in formic acid afforded the formate (**10**) in 79% yield.

We had thus obtained the desired compound **9**, which was converted into **1a** by means of the following sequence of reactions. Reductive debromination of **9** to **11** was easily accomplished by treatment with tributyltin hydride (n-Bu_3SnH)[8] and α,α'-bis(isobutyronitrile) (AIBN) in benzene at 60 °C in 97% yield. Treatment of **11** with anhydrous potassium carbonate in ethanol at room temperature afforded the hydroxy ester (**12**). The hydroxy group in **12** was protected with tetrahydropyranyl ether to give **13**. After reduction of the ester group in **13** with lithium aluminum hydride in tetrahydrofuran (THF), the obtained alcohol (**14**) was oxidized to the aldehyde (**15**) with excess sulfur trioxide (SO_3)–pyridine complex and triethylamine in dimethyl sulfoxide (DMSO). The Wittig reaction of **15** with tributyl 2-oxoheptylidenephosphorane (n-$Bu_3P=CHCOC_5H_{11}$) in ether at room temperature gave the α,β-unsaturated ketone (**16**) in 92% yield from the ester (**13**). Re duction of the ketone group in **16** with $NaBH_4$ in the presence of cerium (III) chloride ($CeCl_3$) in methanol gave the more polar 15α-alcohol (**17a**) (PG numbering) and the less polar 15β-alcohol (**17b**) in 49% and 44% yields, respectively. The stereochemistry at the C_{15} position was tentatively assigned on the basis of the relative thin layer chromatographic (TLC) mobilities (silica gel). In general in prostaglandin chemistry, the more polar alcohol is the 15α-hydroxy compound, and the less polar alcohol is the 15β-hydroxy compound. Protection of the hydroxy group in the 15α-alcohol (**17a**) with dihydropyran (DHP) and p-toluenesulfonic acid in methylene chloride, followed by treatment with excess sodium metal in liquid ammonia at -78 °C gave the alcohol (**19**) in 74% yield from **17a**. Alkylation of the alcohol (**19**) with lithium chloroacetate ($ClCH_2COOLi$) afforded the carboxylic acid (**20**). Finally, removal of the protective groups of **20** with camphorsulfonic acid in aqueous acetone gave the desired compound **1a** as a colorless viscous oil.

By using a sequence of reactions similar to that described for the synthesis of **1a**, the 15β-alcohol (**17b**) was led to the 15β-isomer (**1b**).

Compounds **1a** and **1b** exhibited very weak inhibitory activities (IC_{50}: 5.4 and 4.1 μg/ml, respectively) against adenosine diphosphate induced platelet aggregation using rabbit platelet-rich plasma.

Experimental

Infrared (IR) spectra were recorded with a JASCO A-102 spectrophotometer. ^{1}H-NMR spectra were recorded with a Varian T-60A (60 MHz) or EM-390 (90 MHz) instrument or with a JEOL JNM-DX-270 (270 MHz) spectrometer in deuteriochloroform, with tetramethylsilane as an internal reference. Low-resolution mass spectra (LR-MS) were obtained with a JEOL JMS-01SG or JMS-G300 mass spectrometer and high-resolution mass spectra (HR-MS) with a JEOL JMS-HX100 or JMS-G300 mass spectrometer. Removal of solvents *in vacuo* was accomplished with a rotating flash evaporator at 20—30 mmHg and usually at 35—50 °C. Plates for TLC were Silica gel 60 F-254 (E. Merck AG) and spots were visualized by spraying a solution of 0.5% vanillin in 20% ethanol in sulfuric acid (v/v), followed by heating. Columns for ordinary chromatography were prepared with Silica gel 60 (70—230 mesh or 230—400 mesh, E. Merck AG). In general, reactions were carried out under a nitrogen stream.

(1*R,2*R**,3*R**,5*S**,6*R**,7*R**,8*S**)-7-Acetoxy-3-[2-(benzyloxy)ethyl]-6-ethoxycarbonyl-3-hydroxytricyclo-[3.3.0.0^{2,8}]octane (8)**——$NaBH_4$ (0.712 g) was added to a stirred solution of **6** (3.012 g) in ethanol (30 ml) under

ice-cooling, and the whole was stirred for 2 h. The reaction mixture was poured into brine and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an unstable hydroxy ester (**7**), which was treated with Ac_2O (10 ml) in pyridine (20 ml) for 4 h at room temperature. The reaction mixture was poured into water and extracted with Et_2O. The extracts were washed with dil. HCl and brine, and dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel column chromatography. Elution with 20—35% AcOEt in hexane (v/v) afforded **8** (1.860 g) as a colorless oil. IR (neat): 3500, 1735, 1235 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, J=7 Hz, $OCH_2\underline{CH}_3$), 1.97 (3H, s, CH_3CO), 4.10 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 4.48 (2H, s, CH_2Ph), 5.67 (1H, br d, J=6 Hz, C_7-H), 7.30 (5H, s, arom.-H). LR-MS *m/z*: 370 ($M^+ - H_2O$), 329, 311. HR-MS *m/z*: Calcd for $C_{22}H_{26}O_5$ ($M^+ - H_2O$): 370.1780. Found: 370.1790.

(1*S,5*R**,6*R**,7*S**,8*S**)-7-Acetoxy-3-[2-(benzyloxyl)ethyl]-8-bromo-6-ethoxycarbonylbicyclo[3.2.1]oct-2-ene (9)**——A 48% aqueous solution of HBr (14 ml) was added to a stirred solution of **8** (1.430 g) in Et_2O (50 ml) under ice-cooling, and the whole was stirred for 20 min under the same conditions. The reaction mixture was poured into water and extracted with Et_2O. The extracts were washed with $NaHCO_3$ aq. and brine, and dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel column chromatography. Elution with 6—12% AcOEt in hexane (v/v) afforded **9** (1.050 g) as a colorless oil. IR (neat): 1735, 1240 cm^{-1}. ^{1}H-NMR (270 MHz) ($CDCl_3$) δ: 1.27 (3H, t, J=7.2 Hz, $OCH_2\underline{CH}_3$), 2.01 (3H, s, CH_3CO), 2.74 (1H, br d, J=5.8 Hz, C_6-H), 3.16 (1H, br t, J=5.8 Hz, C_1-H), 3.52 (2H, t, J=6.6 Hz, $OC\underline{H}_2CH_2$), 4.21 (2H, q, J=7.2 Hz, $OC\underline{H}_2CH_3$), 4.34 (1H, br s, C_8-H), 4.49 (2H, s, CH_2Ph), 5.37 (1H, br d, J=5.8 Hz, C_2-H), 5.93 (1H, t, J=5.8 Hz, C_7-H), 7.32 (5H, s, arom.-H). LR-MS *m/z*: 452 (M^++2), 450 (M^+), 344, 346. HR-MS *m/z*: Calcd for $C_{22}H_{27}BrO_5$ (M^++2): 452.1022. Found: 452.1012.

(1*S,5*R**,6*R**,7*S**,8*S**)-7-Acetoxy-3-[2-(benzyloxy)ethyl]-6-ethoxycarbonyl-8-formyloxybicyclo[3.2.1]oct-2-ene (10)**——A 48% aqueous solution of HBr (0.1 ml) was added to a stirred solution of **8** (192 mg) in 99% formic acid (2 ml) at room temperature, and the whole was stirred for 30 min under the same conditions. The reaction mixture was poured into water and extracted with Et_2O. The extracts were washed with $NaHCO_3$ aq. and brine, then dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel column chromatography. Elution with 14—18% AcOEt in hexane (v/v) afforded **10** (162 mg) as a colorless oil. IR (neat): 1730, 1240, 1165 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, J=7 Hz, $OCH_2\underline{CH}_3$), 1.99 (3H, s, CH_3CO), 3.50 (2H, t, J=6 Hz, $OC\underline{H}_2CH_2$), 4.11 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 4.46 (2H, s, CH_2Ph), 5.01 (1H, br s, C_8-H), 5.32 (1H, br d, J=6 Hz, C_2-H), 5.52 (1H, t, J=6 Hz, C_7-H), 7.26 (5H, s, arom.-H), 7.89 (1H, s, CHO). LR-MS *m/z*: 416 (M^+), 312, 220.

(1*S,5*S**,6*R**,7*R**)-7-Acetoxy-3-[2-(benzyloxy)ethyl]-6-ethoxycarbonylbicyclo[3.2.1]oct-2-ene (11)**——A mixture of **9** (1.050 g) and *n*-Bu_3SnH (1.0 ml) and AIBN (50 mg) in toluene (30 ml) was stirred for 30 min at 60 °C. The reaction mixture was concentrated to dryness. The residue obtained was purified by silica gel column chromatography. Elution with 6—9% AcOEt in hexane (v/v) afforded **11** (840 mg) as a colorless oil. IR (neat): 1735, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7 Hz, $OCH_2\underline{CH}_3$), 1.97 (3H, s, CH_3CO), 3.51 (2H, t, J=6 Hz, $OC\underline{H}_2CH_2$), 4.13 (2H, q, J=6 Hz, $OC\underline{H}_2CH_3$), 4.48 (2H, s, CH_2Ph), 5.10 (1H, t, J=6 Hz, C_7-H), 5.52 (1H, br d, J=6 Hz, C_2-H), 7.30 (5H, s, arom.-H). LR-MS *m/z*: 372 (M^+), 312, 266. HR-MS *m/z*: Calcd for $C_{22}H_{28}O_5$ (M^+): 372.1937. Found: 372.1931.

(1*S,5*S**,6*R**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-ethoxycarbonyl-7-hydroxybicyclo[3.2.1]oct-2-ene (12)**——A mixture of **11** (840 mg) and anhydrous K_2CO_3 (2.00 g) in ethanol (20 ml) was stirred for 3 h at room temperature. The reaction mixture was poured into water and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 12—18% AcOEt in hexane (v/v) afforded **12** (715 mg) as a colorless oil. IR (neat): 3440, 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, t, J=7 Hz, $OCH_2\underline{CH}_3$), 3.48 (2H, t, J=6 Hz, $OC\underline{H}_2CH_2$), 4.10 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 4.45 (2H, s, OCH_2Ph), 5.55 (1H, br d, J=6 Hz, C_2-H), 7.36 (5H, s, arom.-H). LR-MS *m/z*: 330 (M^+), 312. HR-MS *m/z*: Calcd for $C_{20}H_{26}O_4$ (M^+): 330.1836. Found: 330.1840.

(1*S,5*S**,6*R**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-ethoxycarbonyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.2.1]oct-2-ene (13)**——A mixture of **12** (714 mg), DHP (0.30 ml) and a catalytic amount of *p*-TsOH in CH_2Cl_2 (20 ml) was stirred under ice-cooling for 30 min. The reaction mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ aq. and brine, and then dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 4—7% AcOEt in hexane (v/v) afforded **13** (839 mg) as a colorless oil. IR (neat): 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.24 (3H, t, J=7 Hz, $OCH_2\underline{CH}_3$), 4.12 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 4.48 (2H, s, OCH_2Ph), 4.60 (1H, br s, OCHO), 5.60 (1H, br t, J=6 Hz, C_2-H), 7.30 (5H, s, arom.-H). LR-MS *m/z*: 414 (M^+), 330. HR-MS *m/z*: Calcd for $C_{25}H_{34}O_5$ (M^+): 414.2406. Found: 414.2383.

(1*S,5*S**,6*S**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-hydroxymethyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.2.1]oct-2-ene (14)**——A solution of **13** (826 mg) in THF (8 ml) was added to a stirred suspension of $LiAlH_4$ (120 mg) in THF (15 ml) under ice-cooling, and the whole was stirred for 30 min, and then quenched with 4% NaOH aq. (0.5 ml). The reaction mixture was stirred at room temperature for another 1 h, and then the precipitate was filtered off. Removal of the solvent of the filtrate *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 15—25% AcOEt in hexane (v/v) afforded **14** (723 mg) as a colorless oil. IR (neat): 3470, 1020 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.47 (2H, s, OCH_2Ph), 5.57 (1H, br t, J=6 Hz, C_2-H), 7.28 (5H, s, arom.-H). LR-MS *m/z*: 372

(M^+), 354, 288. HR-MS *m/z*: Calcd for $C_{23}H_{32}O_4$ (M^+): 372.2301. Found: 372.2291.

(1*S,5*S**,6*R**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-formyl-7-(tetrahydropyran-2-yl)oxybicyclo[3.2.1]oct-2-ene (15)**——A solution of sulfur trioxide–pyridine complex (2.10 g) in DMSO (15 ml) was added to a stirred mixture of **14** (720 mg) and Et_3N (6.7 ml) in DMSO (7 ml) at room temperature. After being stirred for 30 min, the reaction mixture was poured into ice-water and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a practically pure aldehyde (**15**) (691 mg) as a pale yellow oil. The crude material was used for the subsequent step without purification. IR (neat): 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.46 (2H, s, OCH_2Ph), 4.58 (1H, br s, OCHO), 5.57 (1H, br t, J=6 Hz, C_2-H), 7.25 (5H, s, arom.-H), 9.70 (1H, d, J=4 Hz, CHO).

(1*S,5*S**,6*S**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-[3-oxo-1(*E*)-octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo[3.2.1]-oct-2-ene (16)**——Tributyl 2-oxoheptylidenephosphorane (840 mg) in Et_2O (5 ml) was added to a solution of **15** (688 mg) in Et_2O (15 ml), and the whole was stirred for 2.5 h at room temperature. The reaction mixture was concentrated to dryness. The resultant residue was purified by silica gel column chromatography. Elution with 6—9% AcOEt in hexane (v/v) afforded **16** (850 mg) as a colorless oil. IR (neat): 1690, 1670, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.48 (2H, s, OCH_2Ph), 5.60 (1H, br t, J=6 Hz, C_2-H), 6.09 (1H, dd, J=3, 16 Hz, olefinic-H), 6.60—7.10 (1H, m, olefinic-H), 7.30 (5H, s, arom.-H). LR-MS *m/z*: 382 (M^+-84), 364. HR-MS *m/z*: Calcd for $C_{25}H_{34}O_3$ ($M^+-C_5H_8O$): 382.2508. Found: 382.2505.

(1*S,5*S**,6*S**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-[3(*S**)-hydroxy-1(*E*)octenyl]-7-(tetrahydropyran-2-yl)oxybicyclo-[3.2.1]oct-2-ene (17a) and (1*S**,5*S**,6*S**,7*R**)-3-[2-(Benzyloxy)ethyl]-6-[3(*R**)-hydroxy-1(*E*)-octenyl]-7-(tetra-hydropyran-2-yl)oxybicyclo[3.2.1]oct-2-ene (17b)**——$NaBH_4$ (100 mg) was added to a stirred solution of **16** (846 mg) and $CeCl_3 \cdot 7H_2O$ (812 mg) in methanol (16 ml) under ice-cooling. After 30 min of stirring, the excess reagent was decomposed by addition of AcOH, and the reaction mixture was diluted with brine and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 10% AcOEt in hexane (v/v) afforded **17b** (376 mg) as a colorless oil, and further elution with 12% AcOEt in hexane (v/v) afforded **17a** (420 mg) as a colorless oil. Compound **17a**: IR (neat): 3450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.52 (2H, s, OCH_2Ph), 4.68 (1H, br s, OCHO), 5.30—5.90 (3H, m, olefinic-H), 7.35 (5H, s, arom.-H). LR-MS *m/z*: 366 (M^+-102), 276. HR-MS *m/z*: Calcd for $C_{25}H_{34}O_2$ ($M^+-C_5H_{10}O_2$): 366.2559. Found: 366.2542. Compound **17b**: IR (neat): 3450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.52 (2H, s, OCH_2Ph), 4.70 (1H, br s, OCHO), 5.30—5.90 (3H, m, olefinic-H), 7.35 (5H, s, arom.-H). LR-MS *m/z*: 366 (M^+-102), 276. HR-MS *m/z*: Calcd for $C_{25}H_{34}O_2$ ($M^+-C_5H_{10}O_2$): 366.2559. Found: 366.2558.

(1*S,5*S**,6*S**,7*R**)-3-[2-(Benzyloxy)ethyl]-7-(tetrahydropyran-2-yl)oxy-6-[3(*S**)-(tetrahydropyran-2-yl)oxyoct-1(*E*)-enyl]bicyclo[3.2.1]oct-2-ene (18)**——A mixture of **17a** (410 mg), DHP (0.12 ml) and a catalytic amount of *p*-TsOH in CH_2Cl_2 (4 ml) was stirred under ice-cooling for 15 min. The reaction mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ aq. and brine, and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by silica gel column chromatography. Elution with 5—9% AcOEt in hexane (v/v) afforded **18** (410 mg) as a colorless oil. IR (neat): 2950, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.47 (2H, s, OCH_2Ph), 4.62 (2H, br s, OCHO), 5.10—5.90 (3H, m, olefinic-H), 7.27 (5H, s, arom.-H). LR-MS *m/z*: 366 ($M^+-102-84$), 285. HR-MS *m/z*: Calcd for $C_{25}H_{34}O_2$ ($M^+-C_5H_{10}O_2-C_5H_8O$): 366.2559. Found: 366.2533.

(1*S,5*S**,6*S**,7*R**)-3-(2-Hydroxyethyl)-7-(tetrahydropyran-2-yl)oxy-6-[3(*S**)-(tetrahydropyran-2-yl)oxyoct-1(*E*)-enyl]bicyclo[3.2.1]oct-2-ene (19)**——Excess sodium metal was added to a stirred solution of **18** (402 mg) in a mixture of liquid ammonia (20 ml) and THF (14 ml) at −78 °C until a blue color persisted, and the whole was stirred for 20 min. The reaction was quenched by the addition of NH_4Cl, and then ammonia was evaporated off at room temperature under a stream of N_2. Water was added to the residue and the mixture was extracted with Et_2O. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent gave an oily residue, which was purified by silica gel column chromatography. Elution with 12—16% AcOEt in hexane (v/v) afforded **19** (294 mg) as a colorless oil. IR (neat): 3500 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.65 (2H, br s, OCHO), 5.10—5.90 (3H, m, olefinic-H). LR-MS *m/z*: 360 (M^+-102), 316. HR-MS *m/z*: Calcd for $C_{23}H_{36}O_3$ ($M^+-C_5H_{10}O_2$): 360.2664. Found: 360.2648.

(1*S,5*S**,6*S**,7*R**)-3-[2-(Carboxymethoxy)ethyl]-7-(tetrahydropyran-2-yl)oxy-6-[3(*S**)-(tetrahydropyran-2-yl)oxyoct-1(*E*)-enyl]bicyclo[3.2.1]oct-2-ene (20)**——A solution of 15% *n*-BuLi in hexane (0.53 ml) was added to a stirred solution of **19** (282 mg) in THF (7 ml) under ice-cooling. The mixture was stirred for 10 min, then dimethylformamide (1.0 ml), DMSO (1.0 ml), $ClCH_2COOLi$ (200 mg) and NaI (500 mg) were added, and the whole was stirred for 5 h at room temperature. The reaction mixture was diluted with water, acidified with 3% HCl aq. and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified by acid-washed silica gel column chromatography. Elution with 20—30% AcOEt in hexane (v/v) afforded **20** (220 mg) as a colorless oil. IR (neat): 1755, 1740 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.06 (2H, s, OCH_2COOH), 4.66 (2H, br s, OCHO), 5.10—5.90 (3H, m, olefinic-H), 8.58 (1H, s, COOH). LR-MS *m/z*: 334 ($M^+-102-84$), 258, 154. HR-MS *m/z*: Calcd for $C_{20}H_{32}O_5$ ($M^+-C_5H_{10}O_2-C_5H_8O$): 334.2144. Found: 334.2154.

(1*S,5*S**,6*S**,7*R**)-3-[2-(Carboxymethoxy)ethyl]-7-hydroxy-6-[3(*S**)-hydroxyoct-1(*E*)-enyl]bicyclo[3.2.1]oct-2-ene (1a)**——A mixture of **20** (216 mg) and camphorsulfonic acid (20 mg) in acetone (8 ml) and water (4 ml) was stirred at 40 °C for 2 h. The reaction mixture was poured into water and extracted with AcOEt. The extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave an oily residue, which was purified

by acid-washed silica gel column chromatography. Elution with 70% AcOEt in hexane (v/v) to AcOEt afforded **1a** (97 mg) as a colorless viscous oil. IR (neat): 3400, 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.67 (2H, t, J=6 Hz, $OC\underline{H}_2CH_2$), 4.07 (2H, s, $OC\underline{H}_2COOH$), 5.40—5.85 (3H, m, olefinic-H). LR-MS m/z: 334 (M^+-18), 316, 290. HR-MS m/z: Calcd for $C_{20}H_{32}O_5$ (M^+-H_2O): 334.2144. Found: 334.2132. Rf=0.27 (benzene : AcOEt : MeOH : AcOH = 20 : 60 : 1 : 1).

(1*S,5*S**,6*S**,7*R**)-3-[2-(Carboxymethoxy)ethyl]-7-hydroxy-6-[3(*R**)-hydroxyoct-1(*E*)-enyl]bicyclo[3.2.1]oct-2-ene (1b)**——Similar treatment of the 15β-alcohol (**17b**) through the reaction sequence used for the synthesis of **1a** gave **1b** as a colorless viscous oil. IR (neat): 3400, 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.67 (2H, t, J=6 Hz, $OC\underline{H}_2CH_2$), 4.09 (2H, s, $OC\underline{H}_2COOH$), 5.40—5.85 (3H, m, olefinic-H). LR-MS m/z: 334 (M^+-18), 316, 290. HR-MS m/z: Calcd for $C_{20}H_{32}O_5$ (M^+-H_2O): 334.2144. Found: 334.2126. Rf=0.36 (benzene : AcOEt : MeOH : AcOH = 20 : 60 : 1 : 1).

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Studies on 3-Aminoindazoles. I. Synthesis of 1- or 3-(Substituted 3-Amino)indazoles

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Various 1- or 3-(substituted 3-amino)indazoles with anti-inflammatory effects were synthesized by means of three methods, as follows. 1) Reactions of 3-aminoindazole (**1**) with acrylamides (**2a** and **2b**) gave amide derivatives (**3a** and **3b**) having a carbamoylethylamino group at the 3-position of **3a** and **3b**. The amide derivatives (**3a** and **3b**) were converted to thioamide derivatives (**4a** and **4b**) by treatment with P_2S_5. Electrode reduction of **4a** and **4b** gave 3-(substituted 3-amino)indazoles (**5a** and **5b**). 2) The reaction of **1** with aminoalkyl halides (**6c—r**) gave 3-(substituted 3-amino)indazoles (**5c—r**) and 1-(substituted 3-amino)indazoles (**7c—r**) in a ratio of 3 : 1. 3) The reaction of **1** with phthalic anhydride (**8**) gave 3-phthalimidoindazole (**9**). Compound **9** was allowed to react with aminoalkyl halides (**6o—r**) to give 1-substituted derivatives (**10s—z**) of **9**. Reactions of **10a—z** with hydrazine hydrate gave 1-(substituted 3-amino)indazole derivatives (**5s—z**).

Keywords——3-aminoindazole; anti-inflammatory drug; Michael reaction; thioamide; electrode reduction; aminoalkylation

The electrode reactions[1] of organic compounds form a growing and interesting subject in the field of organic synthesis. Although the electrode reduction[2] of thioamide derivatives has been studied, there have been few examples of drug syntheses by means of electrode reactions. We found that 1- or 3-(substituted 3-amino)indazoles are valuable basic non-steroidal anti-inflammatory drugs with analgesic effect, showing reduced side effects[3] (digestive tract ulceration) as compared with acidic non-steroidal anti-inflammatory drugs. In our preceding paper,[4] the anti-inflammatory effect of 3-[3-(2,6-dimethylpiperidino)-

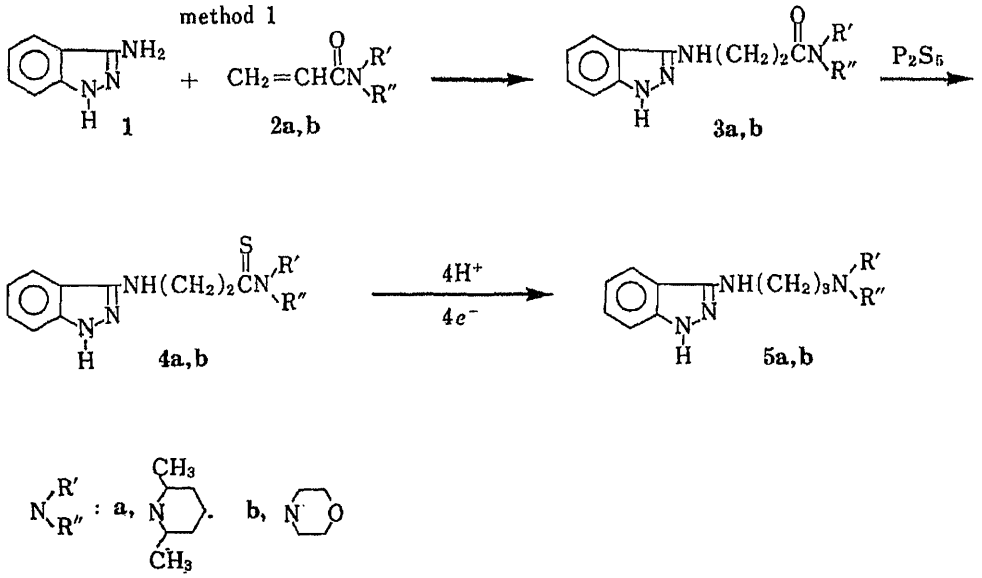


Chart 1

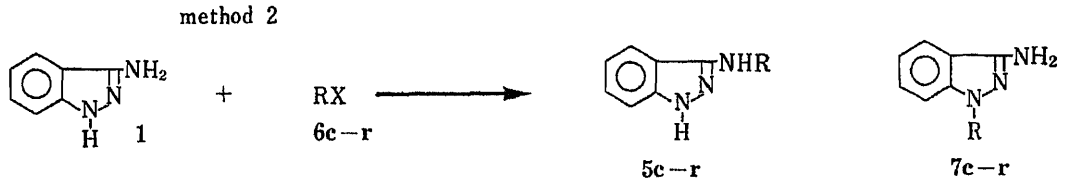


Chart 2

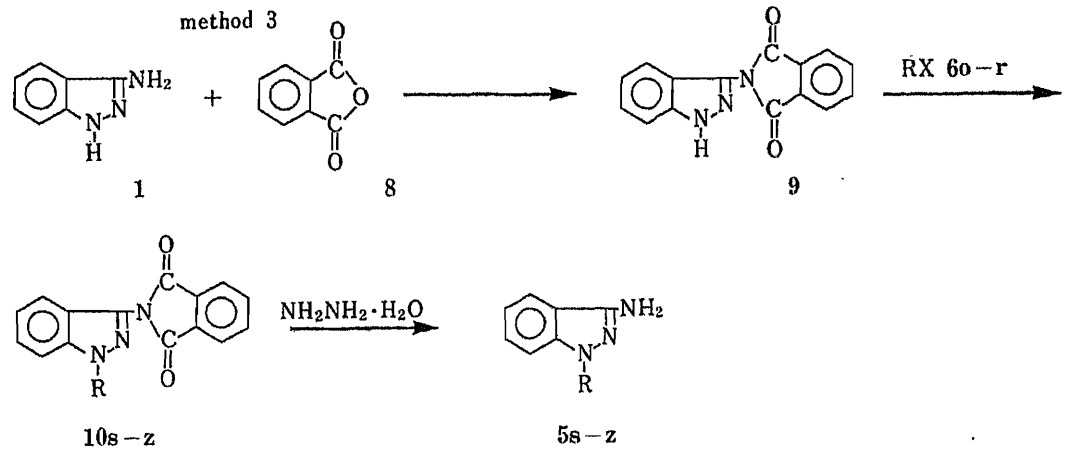
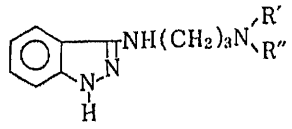
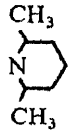


Chart 3

TABLE I. Synthesis of 3-Aminoindazole Derivatives by Electrode Reduction



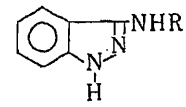
Compd. No.	$N<^{R'}_{R''}$	Synthetic method[a]	Yield[b] (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
5a		1	52	163—164	$C_{17}H_{26}N_4$	71.29 (71.35	9.15 8.93	19.56 19.72)
5b		1	51	155—156	$C_{14}H_{20}N_4O$	64.59 (64.47	7.74 7.74	21.52 21.52)

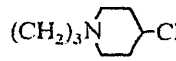
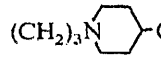
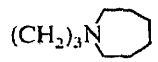
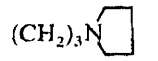
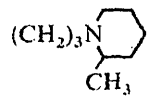
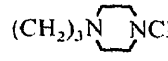
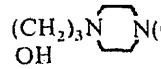
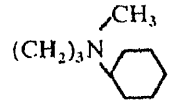
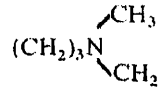
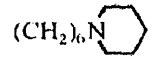
a) See Chart 1. *b*) Overall yield.

propylamino]indazole (**5a**) was reported. We wish to describe here a selective electrode reduction of thioamide compounds representing an interesting application of electro-organic chemistry to drug synthesis. The methods used for the preparation of 1-(substituted 3-amino)indazoles and 3-(substituted 3-amino)indazoles (**5a**—**z**) are summarized in Charts 1—3.

As part of a seach for more effective compounds, we synthesized some derivatives (**5a**—**z**) as follows: (1) introduction of an aminoalkyl moiety into C_3-NH_2 as summarized in Tables I—V; (2) introduction of an aminoalkyl moiety into N(1) as summarized in Tables VI and VII.

TABLE II. Synthesis of 3-Aminoindazole Derivatives by Aminoalkylation



Compd. No.	R	Synthetic method[a]	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)			
						C	H	Cl	N
5c	$(CH_2)_3N(CH_2CH_3)_2$	2	52	81—82.5	$C_{14}H_{22}N_4$	68.26 (67.98	9.00 9.25		22.74 22.77)
5d		2	59	171—173	$C_{15}H_{21}ClN_4$	61.53 (61.49	7.23 7.39	12.11 12.14	19.13 18.98)
5e		2	53	160—161	$C_{15}H_{22}N_4O$	65.67 (65.81	8.08 7.92		20.42 20.39)
5f		2	55	163—165	$C_{16}H_{24}N_4$	70.55 (70.67	8.88 9.01		20.57 20.32)
5g	$(CH_2)_3N(n\text{-}Bu)_2$	2	56	101—103	$C_{18}H_{30}N_4$	71.48 (71.62	10.00 9.78		18.52 18.60)
5h		2	54	163—165	$C_{14}H_{20}N_4$	68.82 (69.01	8.25 8.03		22.93 22.96)
5i		2	49	96—99	$C_{16}H_{24}N_4$	70.55 (70.61	8.88 9.02		20.57 20.37)
5j		2	57	110—116	$C_{15}H_{23}N_5$	65.90 (66.11	8.48 8.27		25.62 25.62)
5k		2	62	69—74	$C_{16}H_{25}N_5O$	63.34 (63.21	8.30 8.45		23.09 22.99)
5l		2	51	99—102	$C_{17}H_{26}N_4$	71.29 (70.99	9.15 9.38		19.56 19.63)
5m		2	55	127—131	$C_{18}H_{22}N_4$	73.44 (73.11	7.53 7.77		19.03 19.12)
5n		2	59	135—138	$C_{18}H_{28}N_4$	71.96 (72.02	9.39 9.21		18.65 18.77)

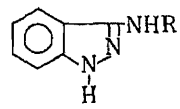
a) See Chart 2.

As an example of (1), a Michael addition of 3-aminoindazole[5] (**1**) to *N*-acryloyl-2,6-dimethylpiperidine (**2a**) in a non-solvent gave 3-[(2,6-dimethylpiperidino)carbonylethylamino]indazole (**3a**) after 2 h at 80 °C, in a yield of 77%.

The first step of the reaction shown in Chart 1 requires selective Michael alkylation (method 1) at C_3-NH_2 of **1**. As shown in Chart 2, a simple alkylation (method 2) of **1** with 1-(3-bromopropyl)-2,6-dimethylpiperidinium bromide[6] and sodium carbonate in dimethylformamide (DMF) at 80 °C for 1 d gave a positionally isomeric mixture of the N(1)-alkyl derivative and the C_3-NH-alkyl derivative in a ratio of 1 : 3. The present Michael addition is quite suitable for selective preparations of **3a** and **3b**.

Compounds **3a** and **3b** reacted with P_2S_5 in dioxane for 2 h at 60 °C to give the thioamide derivatives (**4a** and **4b**) in a yield of 60—80%. Compounds **4a** and **4b** were converted to the target molecules (**5a** and **5b**) by electrode reduction under the following conditions. The

TABLE III. Synthesis of 3-Aminoindazole Derivatives by Aminoalkylation



Compd. No.	R	Synthetic method[a]	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
5o	$(CH_2)_2N(CH_3)_2$	2	41	109—112	$C_{11}H_{16}N_4$	64.68 (64.39)	7.89 8.02	27.43 27.59)
5p	$(CH_2)_2N$	2	56	130—132	$C_{14}H_{20}N_4$	68.82 (68.99)	8.25 8.01	22.93 23.00)
5q	$(CH_2)_3NH_2$	2	53	111—114	$C_{10}H_{14}N_4$	63.13 (62.98)	7.42 7.59	29.45 29.43)
5r	$(CH_2)_2$ 	2	62	140—144	$C_{15}H_{15}N_3$	75.92 (76.08	6.37 6.19	17.71 17.73)

a) See Chart 2.

apparatus was composed of two containers which were equipped with two lead electrodes and separated from one another with a cationic exchange membrane of sulfonated divinylbenzene–styrene–butadiene copolymer. Successively, a solution of 3-[(2,6-dimethylpiperidino)thiocarbonylethylamino]indazole (**4a**) in sulfuric acid, water and tetraethylammonium ethyl sulfate solution (24.5%) were added to the cathodic container and the anionic container was filled with 20% aqueous sulfuric acid. The electrolytic reduction was carried out at a current density of 25 mA/cm^2 and a working cathode potential of 3.5 V (*vs.* SCE) at 27—30 °C to give **5a** in a yield of 96%. However the reduction of **3a** with lithium aluminum hydride unexpectedly gave **1** in a yield of 30—40% and the target compound (**5a**) in a yield of 60—70%. An alternative reduction of **4a** with Raney-Ni was also carried out in 80% aqueous dioxane. However, we obtained a poor yield of **5a** (42%), with significant formation of 3-(ethylamino)indazole (24%) and **1** (25%). The present electrode reaction is far more selective and safer than lithium aluminum hydride (LAH) reduction or Raney-Ni reduction.

As an example of (2) (method 3), **1** reacted with phthalic anhydride (**8**) in dioxane for 5 h at 120 °C to give 3-phthalimidoindazole (**9**) in a yield of 87%. Compound **9** was allowed to react with aminoalkyl halides (**6o—r**) in DMF for 12 h at 80 °C to give 1-(substituted aminoalkyl)-3-phthalimidoindazoles (**10s—z**) in a yield of 55—75%. Compounds **10s—z** reacted with hydrazine hydrate in ethyl alcohol for 3 h at 0—5 °C to give 1-(substituted 3-amino)indazoles (**5s—z**).

Thus, we have succeeded in the synthesis of novel 1- or 3-(substituted 3-amino)indazoles (**5a—z**), new types of anti-inflammatory drugs, in a high yield by means of electrode reduction and aminoalkylation.

Experimental

All melting points were determined on a Buchi 510 apparatus and are uncorrected. Infrared (IR) spectra were determined using a Hitachi IR-260-10 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on an NEC JMN GX-400 instrument using tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were measured with an NEC 01-SG mass spectrometer. Column chromatography was carried out on alumina, activated, basic, W-200 (Wako Pure Chemical Industry, Ltd.) and Silica gel 60 (0.063—0.200 mm, Wako Pure Chemical Industry, Ltd.).

TABLE IV. Spectral Data for 3-Aminoindazole Derivatives Obtained by Aminoalkylation

Compd. No.	IR (KBr, cm^{-1})	^{1}H-NMR (5% solution of $CDCl_3$)	MS *m/z*
5c	3310, 3160, 2975, 1620, 1550	0.99 (t, J=5 Hz, 6H), 2.34 (m, 4H), 2.47 (q, J=5 Hz, 4H), 4.27 (t, J=5 Hz, 2H), 5.06 (brs, 2H), 7.10 (m, 4H)	246 (M^+), 174, 146, 132, 112
5d	3400, 3310, 2925, 1620, 1550	1.50 (brs, 4H), 2.11 (m, 3H), 2.95 (m, 6H), 4.25 (t, J=5 Hz, 2H), 5.11 (brs, 2H), 7.15 (m, 4H)	292 (M^+), 257, 174, 146, 132
5e	3400, 3300, 2925, 1630, 1550	1.52 (brs, 5H), 2.10 (m, 3H), 2.98 (m, 6H), 4.23 (t, J=5 Hz, 2H), 5.20 (brs, 2H), 7.20 (m, 4H)	274 (M^+), 175, 146, 132, 112
5f	3340, 3210, 3050, 1630, 1550	1.53 (m, 8H), 2.13 (m, 2H), 2.47 (m, 6H), 4.20 (t, J=5 Hz, 2H), 5.60 (brs, 2H), 7.07 (m, 4H)	272 (M^+), 174, 146, 132, 112
5g	3300, 3170, 2950, 1620, 1550	0.95 (m, 6H), 1.28 (m, 10H), 2.30 (m, 6H), 4.24 (t, J=5 Hz, 2H), 5.17 (brs, 2H), 7.13 (m, 4H)	302 (M^+), 260, 246, 146, 112
5h	3310, 3240, 3070, 1610, 1560	1.74 (m, 6H), 2.50 (m, 6H), 4.33 (t, J=5 Hz, 2H), 5.33 (brs, 2H), 7.10 (m, 4H)	244 (M^+), 187, 132, 112
5i	3390, 3200, 3050, 1630, 1560	1.00 (d, J=6 Hz, 3H), 1.57 (m, 6H), 2.34 (m, 2H), 2.73 (m, 5H), 4.20 (t, J=5 Hz, 2H), 5.21 (brs, 2H), 7.20 (m, 4H)	272 (M^+), 174, 146, 132, 112
5j	3300, 3150, 2940, 1620, 1545	2.30 (m, 7H), 2.50 (m, 8H), 4.13 (t, J=5 Hz, 2H), 5.20 (brs, 2H), 7.20 (m, 4H)	273 (M^+), 258, 230, 174, 132
5k	3400, 2940, 1620, 1545	2.20 (m, 2H), 2.50 (m, 13H), 3.67 (t, J=5 Hz, 2H), 5.11 (brs, 2H), 7.28 (m, 4H)	303 (M^+), 286, 272, 258, 174, 160, 146, 132
5l	3300, 3150, 3050, 2940, 1622, 1550	1.43 (m, 13H), 2.20 (m, 3H), 2.30 (t, J=6 Hz, 2H), 4.30 (t, J=5 Hz, 2H), 5.20 (brs, 2H), 7.20 (m, 4H)	286 (M^+), 271, 175, 161, 153, 147
5m	3350, 3125, 2950, 1650, 1580	2.17 (m, 5H), 3.50 (s, 2H), 3.74 (t, J=5 Hz, 2H), 4.34 (t, J=5 Hz, 2H), 5.05 (brs, 2H), 7.30 (m, 9H)	294 (M^+), 280, 274, 217, 205, 203, 176
5n	3300, 3180, 3060, 1630, 1550	1.40 (m, 14H), 2.32 (m, 6H), 4.14 (t, J=5 Hz, 2H), 5.17 (brs, 2H), 7.27 (m, 4H)	300 (M^+), 240, 226, 132, 117
5o	3320, 3150, 2975, 1620, 1550	2.25 (s, 6H), 2.63 (t, J=6 Hz, 2H), 4.28 (t, J=6 Hz, 2H), 5.27 (brs, 2H), 7.07 (m, 4H)	204 (M^+), 133, 71
5p	3310, 3180, 3070, 2948, 1625, 1560	1.50 (m, 6H), 2.47 (m, 4H), 2.74 (t, J=6 Hz, 2H), 4.35 (t, J=6 Hz, 2H, 5.20 (brs, 2H), 7.05 (m, 4H)	244 (M^+), 132, 116, 104
5q	3320, 3150, 3060, 2960, 1620, 1550	2.01 (brs, 2H), 2.70 (t, J=6 Hz, 2H), 4.30 (t, J=5 Hz, 2H), 5.28 (brs, 2H), 7.21 (m, 4H)	190 (M^+), 174, 160, 146, 132
5r	3320, 3210, 2940, 1615, 1585	3.70 (t, J=6 Hz, 2H), 4.45 (t, J=6 Hz, 2H), 5.20 (brs, 2H), 7.15 (m, 9H)	237 (M^+), 160, 146, 132

A-1. General Procedure for the Preparation of 3-(Substituted 3-Amino)indazole Derivatives by Means of Electrode Reaction (Method 1)——3-[3-(2,6-Dimethylpiperidino)propylamino]indazole (**5a**): A mixture of **1** (9.43 g) and **2a** (12.3 g) was heated at 80 °C while being stirred for 2 h, then methyl alcohol (10 ml) was added to the reaction mixture. White crystals were obtained by filtration and dried to give **3a** (16.2 g) in a yield of 77%. mp 163—164 °C. IR (KBr): 3310, 3210, 3030, 1630, 1550, 1550 cm^{-1}. ^{1}H-NMR [5% solution in $(CD_3)_2SO$] δ: 1.00 (d, J=6 Hz, 6H), 1.17 (m, 6H), 2.03 (m, 2H), 2.63 (m, 4H), 4.17 (t, J=5 Hz, 2H), 5.12 (brs, 2H), 7.17 (m, 4H), MS *m/z*: 300 (M^+), 282, 205, 174, 160, 143.

Compound **3a** (10 g) and phosphorus pentasulfide (3.70 g) were heated at 60 °C for 2 h in dry dioxane. The solvent was evaporated off under reduced pressure, and chloroform (200 ml) and water (100 ml) were added to the residue. The chloroform phase was dried over anhydrous magnesium sulfate and evaporated to give the residue. Then **4a** (7.46 g) was isolated by means of silica gel (150 g) column chromatography in a yield of 71%. mp 168—171 °C. IR (KBr): 3300, 3170, 2930, 1625, 1540 cm^{-1}. ^{1}H-NMR (5% solution in $CDCl_3$) δ: 1.33 (m, 14H), 3.52 (m, 2H), 4.23 (t, J=5 Hz, 2H), 5.23 (brs, 2H), 7.25 (m, 4H). MS *m/z*: 316 (M^+), 282, 205, 174, 160, 143.

The apparatus was composed of two containers which were equipped with two lead electrodes (2×2 cm) and separated from one another with a cationic exchange membrane of sulfonated divinylbenzene–styrene–butadiene copolymer. Successively, a solution of **4a** (0.33 g) in sulfuric acid (2.5 ml), water (47.5 ml) and tetraethylammonium ethyl sulfate solution (20 ml) (24.5%) were added to the cathodic container and the anionic container was filled with

TABLE V. Spectral Data for By-products (7c—r) Obtained by Aminoalkylation

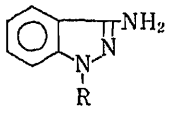
Compd. No.	mp (°C)	IR (KBr, cm^{-1})	^{1}H-NMR (5% solution of $CDCl_3$)	MS *m/z*
7c	66—69	3320, 3150, 2970, 1615, 1545	1.00 (t, J=5 Hz, 6H), 2.27 (m, 4H), 2.46 (q, J=5 Hz, 4H), 4.25 (t, J=5 Hz, 2H), 5.19 (brs, 2H), 7.19 (m, 4H)	246 (M^+), 174, 146, 132, 112
7d	121—123	3300, 3210, 2950, 1610, 1560	1.57 (brs, 4H), 2.18 (m, 3H), 2.99 (m, 6H), 4.26 (t, J=5 Hz, 2H), 5.21 (brs, 2H), 7.25 (m, 4H)	292 (M^+), 257, 174, 146, 132
7e	112—116	3410, 3250, 2920, 1615, 1570	1.59 (brs, 5H), 2.18 (m, 3H), 2.90 (m, 6H), 4.15 (t, J=5 Hz, 2H), 5.25 (brs, 2H), 7.27 (m, 4H)	274 (M^+), 175, 146, 132, 112
7f	99—101	3320, 3200, 3000, 1630, 1540	1.57 (m, 8H), 2.23 (m, 2H), 2.49 (m, 6H), 4.20 (t, J=5 Hz, 2H), 5.56 (brs, 2H), 7.24 (m, 4H)	272 (M^+), 174, 146, 132, 112
7g	88—91	3300, 3100, 2940, 1615, 1540	0.99 (m, 6H), 1.25 (m, 10H), 2.15 (m, 6H), 4.28 (t, J=5 Hz, 2H), 5.27 (brs, 2H), 7.03 (m, 4H)	302 (M^+), 260, 246, 146, 112
7h	111—113	3300, 3200, 3050, 1605, 1550	1.75 (m, 6H), 2.46 (m, 6H), 4.25 (t, J=5 Hz, 2H), 5.33 (brs, 2H), 7.20 (m, 4H)	244 (M^+), 187, 132, 112
7i	97—100	3320, 3210, 2940, 1615, 1580	0.90 (d, J=6 Hz, 3H), 1.50 (m, 6H), 2.32 (m, 7H), 4.00 (brs, 2H), 4.11 (t, J=6 Hz, 2H), 7.18 (m, 4H)	272 (M^+), 257, 173, 160, 146, 132
7j	90—94	3320, 3210, 2940, 1615, 1580	2.32 (m, 15H), 4.15 (t, J=5 Hz, 2H), 4.68 (brs, 2H), 7.33 (m, 4H)	273 (M^+), 258, 173, 160, 146, 132
7k	51—53	3380, 2970, 1625, 1540	2.21 (m, 2H), 2.52 (m, 13H), 3.77 (t, J=5 Hz, 2H), 5.17 (brs, 2H), 7.38 (m, 4H)	303 (M^+), 286, 258, 174, 160, 146, 132
7l	81—83	3320, 3120, 3040, 2930, 1625, 1550	1.46 (m, 13H), 2.30 (m, 3H), 2.35 (t, J=6 Hz, 2H), 4.35 (t, J=5 Hz, 2H), 5.27 (brs, 2H), 7.30 (m, 4H)	286 (M^+), 271, 175, 161, 153, 147
7m	95—97	3300, 3120, 2900, 1660, 1560	2.37 (m, 5H), 3.50 (s, 2H), 3.76 (t, J=5 Hz, 2H), 4.34 (t, J=5 Hz, 2H), 5.35 (brs, 2H), 7.20 (m, 9H)	294 (M^+), 280, 274, 217, 205, 203, 176
7n	93—96	3350, 3150, 3080, 1650, 1550	1.45 (m, 14H), 2.37 (m, 6H), 4.16 (t, J=5 Hz, 2H), 5.37 (brs, 2H), 7.20 (m, 4H)	300 (M^+), 240, 226, 132, 117
7o	75—79	3320, 3200, 2970, 2860, 1610, 1575	2.22 (s, 6H), 2.67 (t, J=6 Hz, 2H), 4.17 (t, J=6 Hz, 2H), 5.06 (brs, 2H), 7.27 (m, 4H)	204 (M^+), 189, 174, 160, 146, 132
7p	101—103	3360, 3100, 3150, 2925, 1615, 1580	1.52 (m, 6H), 2.57 (m, 4H), 2.81 (t, J=6 Hz, 2H), 4.47 (t, J=6 Hz, 2H), 5.65 (brs, 2H), 7.40 (m, 4H)	244 (M^+), 132, 116, 104
7q	64—68	3300, 3130, 3090, 2950, 1630, 1570	2.11 (brs, 2H), 2.75 (t, J=6 Hz, 2H), 4.25 (t, J=5 Hz, 2H), 5.38 (brs, 2H), 7.20 (m, 4H)	190 (M^+), 174, 160, 146, 132
7r	91—93	3430, 3300, 1625, 1615, 1575	3.05 (m, 2H), 4.05 (brs, 2H), 4.33 (t, J=6 Hz, 2H), 7.15 (m, 9H)	238 (M^++1), 160, 146, 132, 117

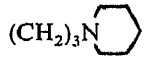
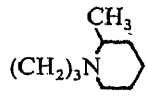
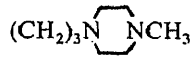
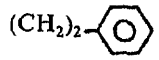
20% sulfuric acid. The electrolytic reduction was carried out at a current density of 25 mA/cm^2 and a working cathode potential of 3.5 V (*vs.* SCE) at 27—30 °C. After 14 h, the pH of the cathodic solution was adjusted to 10 with aqueous sodium hydroxide. The alkaline solution was extracted three times with chloroform (60 ml). The chloroform layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to give **5a** (0.31 g) in a yield of 96%. IR (KBr): 3310, 3210, 3030, 1630, 1550 cm^{-1}. ^{1}H-NMR (5% solution in $CDCl_3$) δ: 1.00 (d, J=6 Hz, 6H), 1.17 (m, 6H), 2.03 (m, 2H), 2.63 (m, 4H), 4.17 (t, J=5 Hz, 2H), 4.37 (brs, 1H), 5.30 (brs, 2H), 7.17 (m, 4H), MS *m/z*: 287, 286 (M^+), 271, 243, 174, 160, 143.

3-(3-Morpholinopropylamino)indazole (**5b**): 3-(3-Morpholinopropylamino)indazole (**5b**) was synthesized in an overall yield of 51% by using *N*-acryloylmorpholine (**2b**) by the same method as described for **5a** (summarized in Table I). IR (KBr): 3200, 2975, 1630, 1615, 1560 cm^{-1}. ^{1}H-NMR (5% solution in $CDCl_3$) δ: 2.10 (m, 4H), 2.34 (m, 4H), 3.70 (m, 4H), 4.30 (t, J=5 Hz, 2H), 5.20 (brs, 2H), 7.20 (m, 4H). MS *m/z*: 261, 260 (M^+), 242, 230, 211, 199, 173, 160.

A-2. The Preparation of 5a by Means of LAH Reduction——A mixture of **3a** (5.77 g) and LAH (1.1 g) in absolute dioxane (300 ml) was heated at 80 °C for 8 h, then cooled to 0 °C in an ice bath. Dioxane (200 ml) was added to the

TABLE VI. Synthesis of 3-Aminoindazole Derivatives by Aminoalkylation



Compd. No.	R	Synthetic method[a]	Yield[b] (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
5s	$(CH_2)_2N(CH_3)_2$	3	38	75—79	$C_{11}H_{16}N_4$	64.68	7.89	27.43
						(64.73	7.59	27.68)
5t		3	32	91—96	$C_{15}H_{22}N_4$	69.73	8.58	21.69
						(70.01	8.31	21.68)
5u		3	33	97—100	$C_{16}H_{24}N_4$	70.55	8.88	20.57
						(70.38	9.01	20.61)
5v		3	36	90—94	$C_{15}H_{23}N_5$	65.90	8.48	25.62
						(65.81	8.52	25.67)
5w	CH_3	3	37	71—72.5	$C_8H_9N_3$	65.29	6.16	28.55
						(64.99	6.27	28.74)
5x	$(CH_2)_2CH_3$	3	34	74—78	$C_{10}H_{13}N_3$	68.54	7.48	23.98
						(68.33	7.62	24.05)
5y	$CH_2CH(CH_3)_2$	3	33	69—73	$C_{11}H_{15}N_3$	69.81	7.99	22.20
						(70.02	7.81	22.17)
5z		3	35	91—93	$C_{15}H_{15}N_3$	75.92	6.37	17.71
						(76.05	6.32	17.63)

a) See Chart 3. *b*) Overall yield.

TABLE VII. Spectral Data for 3-Aminoindazole Derivatives Obtained by Aminoalkylation

Compd. No.	IR (KBr, cm^{-1})	^{1}H-NMR (5% solution of $CDCl_3$)	MS *m/z*
5s	3320, 3200, 2970, 2860, 1610, 1575	2.22 (s, 6H), 2.67 (t, J=6 Hz, 2H), 4.17 (t, J=6 Hz, 2H), 5.06 (brs, 2H), 7.27 (m, 4H)	204 (M^+), 189, 174, 160, 146, 132
5t	3310, 3180, 2940, 1645, 1615	1.43 (m, 6H), 2.17 (m, 8H), 4.25 (t, J=5 Hz, 2H), 5.10 (brs, 2H), 7.20 (m, 4H)	258 (M^+), 173, 160, 146, 132
5u	3320, 3210, 2940, 1615, 1580	0.90 (d, J=6 Hz, 3H), 1.50 (m, 6H), 2.32 (m, 7H), 4.00 (brs, 2H), 4.11 (t, J=6 Hz, 2H), 7.18 (m, 4H)	272 (M^+), 257, 173, 160, 146, 132
5v	3320, 3210, 2940, 1615, 1580	2.32 (m, 15H), 4.15 (t, J=5 Hz, 2H), 4.68 (brs, 2H), 7.33 (m, 4H)	273 (M^+), 258, 173, 160, 146, 132
5w	3170, 2940, 1615, 1560	3.75 (s, 3H), 5.14 (brs, 2H), 7.43 (m, 4H)	147 (M^+), 132, 104
5x	3180, 2930, 1610 1560	0.98 (t, J=5 Hz, 3H), 1.86 (m, 2H), 4.20 (t, J=5 Hz, 2H), 5.15 (brs, 2H), 7.55 (m, 4H)	175 (M^+), 160, 146, 132, 117
5y	3190, 2920, 1620, 1550	0.88 (d, J=6 Hz, 3H), 2.22 (m, 1H), 3.85 (t, J=6 Hz, 2H), 3.98 (brs, 2H), 7.10 (m, 4H)	189 (M^+), 174, 160, 146, 132
5z	3430, 3300, 1625, 1615, 1575	3.05 (m, 2H), 4.05 (brs, 2H), 4.33 (t, J=5 Hz, 2H), 7.15 (m, 9H)	238 (M^+), 160, 146, 132, 117

mixture. Water (1.1 ml) was carefully added dropwise at below 5 °C and 15% sodium hydroxide solution (1.1 ml) was added to the mixture. The mixture was refluxed for 1 h and filtered after cooling. The residue was washed with chloroform (100 ml) and methanol (100 ml). The organic solution was evaporated to dryness, and **5a** (3.38 g) was isolated from the residue by alumina (150 g) column chromatography using chloroform as the solvent in a yield of

61%; **1** was obtained as a by-product in a yield of 35%.

A-3. The Preparation of 5a by Means of Raney-Ni Reduction——A mixture of **4a** (3.0 g) and Raney-Ni (2.87 g) in dioxane (50 ml) and water (12 ml) was heated at 100 °C for 3 h. After that, further Raney-Ni (5.74 g) was added and the mixture was stirred for 4 h. **5a** (1.14 g) was isolated from the residue by alumina (150 g) column chromatography using chloroform as the solvent in a yield of 42%. 3-(Ethylamino)indazole in a yield of 24% and **1** in a yield of 25% were obtained as by-products.

B. General Procedure for the Preparation of 3-(Substituted 3-Amino)indazole Derivatives (5c—r) by Means of Aminoalkylation (Method 2)——A mixture of **1** (4.0 g), 30 mmol amounts of aminoalkyl halide (**6c—r**) and anhydrous potassium carbonate (8.28 g) in DMF (80 ml) was heated for 24 h at 80 °C and then evaporated to dryness. The residue was dissolved in chloroform (100 ml) and the chloroform layer was washed with water (50 ml), dried over anhydrous sodium sulfate and evaporated to dryness. 3-Alkylaminoindazole (**5c—r**) was isolated from the residue by alumina (150 g) column chromatography using chloroform as the solvent. The spectral data are summarized in Tables IV. 1-(Substituted 3-amino)indazoles (**7c—r**) were obtained as by-products in yields of 20—27%, and their spectral data are summarized in Table V.

C. General Procedure for the Preparation of 1-(Substituted 3-Amino)indazole Derivatives (5s—z) by Means of Aminoalkylation (Method 3)——A mixture of **1** (5.0 g) and **8** (6.68 g) in dioxane (50 ml) was stirred for 5 h at 120 °C, then concentrated under reduced pressure. The residue was added to diethyl ether (30 ml) and the mixture was stirred for 30 min to separate crystals, which were filtered off and dried under reduced pressure to give **9** (8.60 g) in a yield of 87%. mp 237—239 °C. IR (KBr): 3310, 1790, 1735, 1625 cm^{-1}. ^{1}H-NMR [5% solution in $(CD_3)_2SO$] δ: 7.57 (m, 8H), 13.35 (br s, 1H). MS *m/z*: 263 (M^+), 236, 219, 207, 192, 179.

A mixture of **9** (5.0 g), 19 mmol amounts of aminoalkyl halide (**6o—r**) and sodium carbonate (2.02 g) in anhydrous DMF (60 ml) was heated for 12 h at 80 °C. After cooling, the mixture was added to water (80 ml), and the reaction product was extracted with diethyl ether (60 ml). The ether layer was extracted three times with 2 N HCl (20 ml). Then the HCl layer was washed with diethyl ether (20 ml) and the pH of the aqueous layer was adjusted to at least 11 with potassium carbonate. The aqueous layer was extracted three times with chloroform (30 ml), then the chloroform layer was dried over anhydrous sodium sulfate, and the chloroform was removed under reduced pressure to give 1-substituted 3-phthalimidoindazoles (**10s—z**) in yields of 50—60%.

Hydrazine hydrate (85%, 2.50 g) was added to a mixture of **10s—z** (8.6 mmol) in ethyl alcohol (70 ml) under cooling with ice. The reaction mixture was stirred for 3 h and then filtered. The filtrate was concentrated under reduced pressure. The residue was added to water (20 ml) and extracted three times with chloroform (20 ml). The chloroform layer was extracted twice with 2 N HCl (20 ml) and the pH of the HCl layer was adjusted to 10 with potassium carbonate. The alkaline layer was extracted three times with chloroform (20 ml). The chloroform extract was dried over anhydrous sodium sulfate, and evaporated under reduced pressure to give 1-(substituted 3-amino)indazoles (**5s—z**). The spectral data are summarized in Table VII.

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Occurrence of Marinobufotoxin and Telocinobufotoxin Homologs in the Skin of *Bufo bankorensis* BORBOUR[1)]

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The occurrence of marinobufagin 3-succinyl-L-arginine and 3-glutaryl-L-arginine esters, and telocinobufagin 3-glutaryl-L-arginine ester, together with seven known bufogenins, in the skin of *Bufo bankorensis* BORBOUR, is reported. The structures were elucidated by degradative means and/or direct comparison with authentic samples. These compounds were assayed for inhibitory activity towards guinea pig heart Na^+, K^+-adenosine triphosphatase.

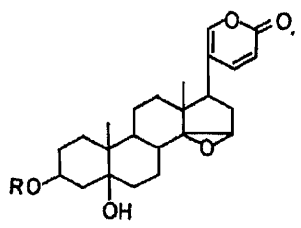
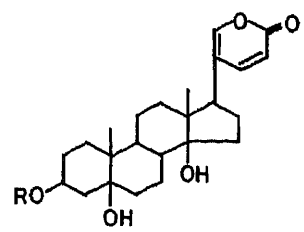
Keywords——*Bufo bankorensis*; toad venom; bufogenin; marinobufotoxin homolog; telocinobufotoxin homolog; sodium-potassium-activated ATPase inhibition

In a series of studies on toad venom,[2)] we characterized the cardiac steroids in the skin of Formosan toad, *Bufo bankorensis* BORBOUR, and assayed them for Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase; EC 3.6.1.3) inhibitory activity.[3)]

Five toads were sacrificed by freezing in dry ice, and the skins were immediately flayed off and extracted with ethanol. The ethanolic extract was concentrated *in vacuo* and the residue was subjected to column chromatography on silica gel. Subsequent separation of the bufogenin and bufotoxin mixture was repeatedly done by high-performance liquid chromatography (HPLC) on a reversed-phase column.

Three new bufotoxins (**5a**, **5b**, **10**) were isolated as colorless amorphous substances, which gave negative ninhydrin and positive Sakaguchi tests. Upon hydrolysis with 6 N hydrochloric acid, arginine was produced and identified by thin-layer chromatography (TLC). When subjected to enzymic hydrolysis with a hog pancreas lipase preparation followed by methylation with diazomethane, these bufotoxins afforded bufogenin 3-hemicarboxylate methyl esters (**3a**, **3b**, **8**). Compounds **3a**, **3b** gave mass spectral (MS) data (*m/z* 382, 364, 213) ascribable to the marinobufagin residue. Pairs of fragment ions (**3a**: *m/z* 133, 115; **3b**: *m/z* 147, 129) suggested the dicarboxylic acid moieties to be succinic acid and glutaric acid monomethyl esters, respectively.[4)] The MS of **8** also showed characteristic fragment ions (*m/z* 384, 366, 323, 147, 129) due to telocinobufagin and a glutaric acid monomethyl ester residue.[4,5)] The absolute configuration of the amino acid residue was elucidated to be L as judged from the substrate specificity of the enzyme used.[2)]

These data prompted us to synthesize marinobufagin 3-succinyl-L-arginine and 3-glutaryl-L-arginine esters (**5a**, **5b**), and telocinobufagin 3-glutaryl-L-arginine ester (**10**) as authentic samples. Marinobufagin (**1**) and telocinobufagin (**6**) were treated with the appropriate dicarboxylic acid anhydride in the presence of 4-(dimethylamino)pyridine[6)] to give marinobufagin 3-hemisuccinate (**2a**) and 3-hemiglutarate (**2b**), and telocinobufagin 3-hemiglutarate (**7**) in satisfactory yields, respectively. Methylation with diazomethane afforded the methyl esters, which showed the same chromatographic behaviors (HPLC and TLC) as the compounds derived from the natural source (**3a**, **3b**, **8**). Treatment of bufogenin 3-hemicarboxylates (**2a**, **2b**, **7**) with *p*-nitrophenol and *N*,*N'*-dicyclohexylcarbodiimide provided the *p*-nitrophenyl esters (**4a**, **4b**, **9**), which in turn were condensed with L-arginine to give the

1: R=H
2: R=CO(CH$_2$)$_n$COOH
3: R=CO(CH$_2$)$_n$COOCH$_3$
4: R=CO(CH$_2$)$_n$COO-C$_6$H$_4$-NO$_2$
5: R=CO(CH$_2$)$_n$CONHCH(CH$_2$)$_3$NHCNH$_2$ (COOH; NH)

6: R=H
7: R=CO(CH$_2$)$_3$COOH
8: R=CO(CH$_2$)$_3$COOCH$_3$
9: R=CO(CH$_2$)$_3$COO-C$_6$H$_4$-NO$_2$
10: R=CO(CH$_2$)$_3$CONHCH(CH$_2$)$_3$NHCNH$_2$ (COOH; NH)

a: n=2, **b**: n=3, **c**: n=4

Chart 1

TABLE I. Inhibition of Na^+,K^+-ATPase by Marinobufotoxin and Telocinobufotoxin Homologs

3-Substituent	Genin	
	Marinobufagin	Telocinobufagin
	747±130[b,11] (1.00)[c]	50.1±7.85 (1.00)
Suc-L-Arg·OH[a]	>6000 (<0.12)	
Glu-L-Arg·OH	2777±340 (0.27)	53.1±1.44 (0.94)
Adi-L-Arg·OH	1537±166[9] (0.49)	
Pim-L-Arg·OH	1237±66 (0.60)	
Sub-L-Arg·OH	427±46.0 (1.75)	33.9±6.25[2] (1.48)

a) Suc=succinyl, glu=glutaryl, adi=adipoyl, pim=pimeloyl, sub=suberoyl. *b*) I_{50}; mean±S.E. (10^{-8} M; n=3). *c*) Figures in parentheses express the potency relative to the respective genin.

desired bufogenin 3-hemicarboxylate-L-arginine esters. The chromatographic behaviors of these synthetic specimens were identical with those of the natural products in HPLC on various reversed-phase columns with different solvent systems. These results support the structures marinobufagin 3-succinyl-L-arginine and 3-glutaryl-L-arginine esters, and telocinobufagin 3-glutaryl-L-arginine ester for **5a**, **5b** and **10**, respectively (Chart 1). Bufalin, resibufogenin, arenobufagin, bufotalin, telocinobufagin, marinobufagin, and gamabufotalin were also isolated and characterized by direct comparison with authentic samples. Lin *et al.*[7] identified resibufogenin, bufotalin, hellebrigenol, and dehydrobufotonin from Formosan "Ch'an Su," a mixture of venoms from *Bufo bufo asiaticus* STEINDACHNER and *Bufo bankorensis* BORBOUR. No further investigation on the venom of the latter toad has been carried out. Recently, we disclosed the existence of cinobufagin 3-glutaryl-L-arginine ester together with other bufotoxin homologs in Korean toad venom.[8] It should be noted that bufotoxin homologs having glutaric acid as a dicarboxylic acid moiety commonly exist in toad venom.

In order to clarify the structure–activity relationship, the naturally occurring bufotoxin homologs and synthetic marinobufagin 3-adipoyl-L-arginine ester (**5c**) were tested for inhibitory activity towards Na^+,K^+-ATPase. The molar concentrations of these compounds giving half-maximal inhibition (I_{50}) of Na^+,K^+-ATPase from guinea pig heart are listed in Table I. Among the marinobufotoxin homologs, the suberoyl-L-arginine ester[9] and succinyl-L-arginine ester exhibited the highest and lowest potencies, respectively. This result is consistent with that obtained for gamabufotalitoxin and cinobufotoxin homologs.[3]

Further studies on cardiac steroids in toad venoms are being conducted in these laboratories, and the details will be reported elsewhere.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. MS measurements were run on a Hitachi M-52 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL FX-90A spectrometer at 90 MHz. Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, and m=multiplet. Silica gel HF_{254} and Silica gel 60 (70—230 mesh) (E. Merck AG, Darmstadt) were used for preparative TLC and column chromatography, respectively. A hog pancreas lipase preparation and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Nakarai Chemicals Ltd. (Kyoto), respectively. HPLC was carried out on a Toyo Soda 803A chromatograph equipped with an ultraviolet detector (280 nm) at a flow rate of 1 ml/min, unless otherwise stated.

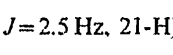
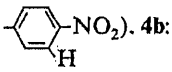
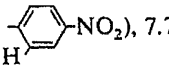
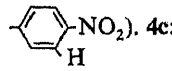
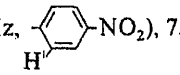
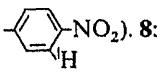
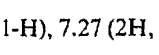
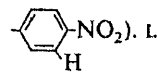
Extraction of Steroidal Components——Five toads (*Bufo bankorensis* BORBOUR) obtained from Vivarium Co. (Tokyo) were sacrificed by freezing in dry ice. The skins were immediately flayed off and extracted with EtOH (200 ml) for 6 months. After removal of insoluble materials by filtration through a Celite layer, the filtrate was concentrated *in vacuo* below 50 °C to give a brown oily residue (116 mg).

Isolation of Bufogenins and Bufotoxins——The residue obtained above was chromatographed on Silica gel 60 (15 cm × 2 cm i.d.) employing hexane–AcOEt (1 : 1) and AcOEt as eluents. Further purification of the dried eluate by HPLC on a Develosil ODS-5 column (Nomura Chem. Co., Seto; 5 μm, 15 cm × 0.4 cm i.d.) with CH_3CN–H_2O (5 : 7, 1 : 2, and 4 : 9) as eluents gave the following bufogenins: bufalin (1 mg), resibufogenin (4 mg), arenobufagin (1 mg), bufotalin (1 mg), telocinobufagin (2 mg), marinobufagin (2 mg), and gamabufotalin (1 mg). These bufogenins were identified by direct comparison with authentic samples (MS, ^{1}H-NMR spectra and chromatographic behavior[4,5,10]).

Further chromatography on silica gel with AcOEt–MeOH (1 : 1) as described above gave a crude bufotoxin mixture, which in turn was subjected to further separation by HPLC on a LiChrosorb RP-18 column (E. Merck AG; 5 μm, 15 cm × 0.4 cm i.d.) with CH_3CN–0.5% $(NH_4)_2CO_3$ (2 : 7) to give two new bufotoxins (t_R **5a**, 7.0 min, 1 mg; t_R **5b**, 10.0 min, 1 mg). Separation on a Develosil ODS-5 column (CH_3CN–H_2O (4 : 11)) also gave a new bufotoxin (t_R **10**, 5.8 min, 1 mg).

Structure Elucidation of New Bufotoxins——All new bufotoxins showed negative ninhydrin and positive Sakaguchi tests. Each new bufotoxin (*ca.* 100 μg) was heated with 6 N HCl (0.2 ml) for 8 h. A portion of the resulting solution was subjected to two-dimensional TLC on Silica gel G (E. Merck AG) using $CHCl_3$–MeOH–17% NH_4OH (2 : 2 : 1) and phenol–H_2O (3 : 1) as developing solvents, and arginine was detected by means of the ninhydrin test. Each new bufotoxin (**5a**, **5b**, **10**, *ca.* 100 μg) obtained from the natural source was dissolved in 1% NaCl–MeOH (9 : 1) (0.5 ml) and incubated with a hog pancreas lipase preparation (*ca.* 500 μg) at 37 °C for 2 h. The incubation mixture was extracted with AcOEt, and the extract was washed with H_2O and then evaporated down *in vacuo*. The residue was dissolved in MeOH (0.05 ml) and treated with ethereal CH_2N_2. After usual work-up, the crude product obtained was subjected to preparative TLC using benzene–AcOEt (1 : 1) as a developing solvent. The adsorbents corresponding to the spots of *Rf* 0.52 (**3a**), 0.61 (**3b**) and 0.22 (**8**) were scraped off and extracted with AcOEt. The products were identical with the methyl esters of synthetic marinobufagin 3-hemisuccinate (**3a**) and 3-hemiglutarate (**3b**), and telocinobufagin 3-hemiglutarate (**8**) with respect to MS and chromatographic behavior. HPLC: LiChrosorb RP-18, CH_3CN–H_2O (1 : 1, t_R **3a**, 4.7 min; **3b**, 5.9 min; **8**, 4.1 min), MeOH–H_2O (2 : 1; t_R **3a**, 4.7 min; **3b**, 5.6 min; **8**, 4.4 min). Develosil ODS-5, CH_3CN–H_2O (1 : 1, t_R **8**, 5.0 min), MeOH–H_2O (2 : 1; t_R **8**, 6.3 min). TLC: benzene–AcOEt (1 : 1: *Rf* **3a**, 0.52; **3b**, 0.61; **8**, 0.22). New bufotoxins (**5a**, **5b**, **10**) obtained from the natural source showed the same chromatographic behavior as the synthetic samples. HPLC: LiChrosorb RP-18, CH_3CN–0.5% $(NH_4)_2CO_3$ (2 : 7; t_R **5a**, 7.8 min; **5b**, 10.7 min) (4 : 15; t_R **10**, 14.5 min). MeOH–0.5% $(NH_4)_2CO_3$ (1 : 1; t_R **5a**, 4.5 min; **5b**, 5.5 min) (4 : 5; t_R **10**, 7.7 min). Develosil ODS-5, CH_3CN–0.5% $(NH_4)_2CO_3$ (2 : 5; t_R **5a**, 4.0 min; **5b**, 5.1 min) (4 : 15; t_R **10**, 17.5 min). MeOH–0.5% $(NH_4)_2CO_3$ (5 : 4; t_R **5a**, 4.2 min; **5b**, 5.0 min) (4 : 5, t_R **10**, 14.2 min). TSKgel ODS-80TM (Toyo Soda Co., 5 μm, 15 cm × 0.4 cm i.d.), CH_3CN–0.5% $(NH_4)_2CO_3$ (1 : 3; t_R **5a**, 13.5 min; **5b**, 18.0 min). MeOH–0.5% $(NH_4)_2CO_3$ (1 : 1; 0.8 ml/min; t_R **5a**, 10.0 min; **5b**, 12.2 min).

Procedure for the Preparation of Bufotoxin Homologs——Each bufogenin (**1**, **6**; 30 mg) was refluxed with succinic

anhydride, glutaric anhydride, or adipic anhydride (17 mg) and 4-(dimethylamino)pyridine (56 mg) in pyridine (2 ml) for 5 h. An additional amount of dicarboxylic acid anhydride (17 mg) was added to the mixture every 1 h. After extraction with AcOEt, the organic layer was washed with 5% HCl, H_2O and dried over anhydrous Na_2SO_4. After evaporation of the solvent, a portion (1 mg) of the residue (30 mg) was redissolved in MeOH (0.1 ml) and treated with CH_2N_2 in the usual manner. The crude product was purified by preparative TLC using benzene–AcOEt (1 : 1) as a developing solvent. The adsorbents corresponding to the spots of *Rf* 0.52 (**3a**), 0.61 (**3b**), 0.63 (**3c**), and 0.22 (**8**) were extracted with AcOEt. The dried extracts showed the following MS data. **3a**: *m/z* 514, 382, 364, 213, 133, 115. **3b**: *m/z* 528, 382, 364, 213, 147, 129. **3c**: *m/z* 542, 382, 364, 213, 161, 143. **8**: *m/z* 530, 384, 366, 323, 147, 129. A solution of each bufogenin 3-hemicarboxylate (30 mg) in AcOEt (2 ml) was treated with *p*-nitrophenol (30 mg) and *N,N'*-dicyclohexylcarbodiimide (50 mg), and the reaction mixture was allowed to stand at room temperature for 6 h. After removal of the precipitate by filtration, the filtrate was evaporated down *in vacuo* and the residue was subjected to preparative TLC. The adsorbents corresponding to the spots of *Rf* 0.51 (**4a**: benzene–AcOEt (1 : 1)), 0.61 (**4b**: benzene–AcOEt (1 : 1)), 0.41 (**4c**: benzene–AcOEt (2 : 1)) and 0.55 (**8**: benzene–AcOEt (1 : 4)) were scraped off and extracted with AcOEt. The dried extracts showed the following ^{1}H-NMR spectral data in $CDCl_3$. **4a**: δ 0.78 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.85 (4H, m, $CO(CH_2)_2CO$), 3.51 (1H, s, 15α-H), 5.30 (1H, brs, $W_{1/2}$=7 Hz, 3α-H), 6.25 (1H, d, J=9.8 Hz, 23-H), 7.22 (1H, d, J=2.5 Hz, 21-H), 7.30 (2H, d, J=9.6 Hz, ), 7.78 (1H, dd, J=9.8, 2.5 Hz, 22-H), 8.28 (2H, d, J=9.6 Hz, ). **4b**: δ 0.78 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.53 (1H, s, 15α-H), 5.29 (1H, brs, $W_{1/2}$=7 Hz, 3α-H), 6.25 (1H, d, J=9.8 Hz, 23-H), 7.22 (1H, d, J=2.5 Hz, 21-H), 7.30 (2H, d, J=9.6 Hz, ), 7.78 (1H, dd, J=9.8, 2.5 Hz, 22-H), 8.28 (2H, d, J=9.6 Hz, ). **4c**: δ 0.78 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 3.51 (1H, s, 15α-H), 5.28 (1H, brs, $W_{1/2}$=7 Hz, 3α-H), 6.24 (1H, d, J=9.8 Hz, 23-H), 7.21 (1H, d, J=2.5 Hz, 21-H), 7.29 (2H, d, J=9.6 Hz, ), 7.78 (1H, dd, J=9.8, 2.5 Hz, 22-H), 8.27 (2H, d, J=9.6 Hz, ). **8**: δ 0.74 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 5.26 (1H, brs, $W_{1/2}$=8 Hz, 3α-H), 6.22 (1H, d, J=9.8 Hz, 23-H), 7.18 (1H, d, J=2.6 Hz, 21-H), 7.27 (2H, d, J=9.5 Hz, ), 7.76 (1H, dd, J=9.8, 2.6 Hz, 22-H), 8.20 (2H, d, J=9.5 Hz, ). L-Arginine (20 mg) in H_2O (1 ml) was added to a solution of each active ester (20 mg) in pyridine (1 ml), and the reaction mixture was allowed to stand at room temperature for 12 h. The resulting solution was diluted with H_2O (100 ml) and percolated through a column packed with Amberlite XAD-4 resin (18 cm × 1.1 cm i.d.). After washing of the column with water (400 ml), the desired compound was eluted with MeOH (100 ml) and dried. The residue obtained was subjected to column chromatography (15 cm × 0.6 cm i.d.) on silica gel with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5). The dried eluate was recrystallized from MeOH–ether to give the corresponding bufotoxin homolog as a white amorphous substance: **5a** (24 mg). mp 195—200 °C (dec.). $[\alpha]_D^{21}$ +24.0° (c=0.13, MeOH). *Anal.* Calcd for $C_{34}H_{48}N_4O_9 \cdot 1/2H_2O$: C, 59.72; H, 7.52; N, 8.19. Found: C, 59.55; H, 7.70; N, 7.73. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.80 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.60 (4H, brs, $W_{1/2}$=7.2 Hz, $CO(CH_2)_2CO$), 3.55 (1H, s, 15α-H), 5.19 (1H, brs, $W_{1/2}$=8 Hz, 3α-H), 6.25 (1H, d, J=9.8 Hz, 23-H), 7.28 (1H, d, J=2.5 Hz, 21-H), 7.82 (1H, dd, J=9.8, 2.5 Hz, 22-H). **5b** (20 mg). mp 183—190 °C (dec.). $[\alpha]_D^{22}$ +15.1° (c=0.13, MeOH). *Anal.* Calcd for $C_{35}H_{50}N_4O_9 \cdot H_2O$: C, 61.03; H, 7.61; N, 8.13. Found: C, 60.75; H, 7.69; N, 7.86. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.76 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.54 (1H, s, 15α-H), 5.19 (1H, brs, $W_{1/2}$=8 Hz, 3α-H), 6.26 (1H, d, J=9.8 Hz, 23-H), 7.27 (1H, d, J=2.5 Hz, 21-H), 7.78 (1H, dd, J=9.8, 2.5 Hz, 22-H). **5c** (20 mg). mp 183—186 °C (dec.). $[\alpha]_D^{23}$ +36.0° (c=0.13, MeOH). *Anal.* Calcd for $C_{36}H_{52}N_4O_9 \cdot 3/2H_2O$: C, 60.74; H, 7.79; N, 7.87. Found: C, 60.97; H, 7.73; N, 7.59. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.79 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.57 (1H, s, 15α-H), 5.23 (1H, brs, $W_{1/2}$=8 Hz, 3α-H), 6.27 (1H, d, J=9.8 Hz, 23-H), 7.30 (1H, d, J=2.5 Hz, 21-H), 7.87 (1H, dd, J=9.8, 2.5 Hz, 22-H). **10** (21 mg). mp 180—185 °C (dec.). $[\alpha]_D^{19}$ +15.4° (c=0.13, MeOH). *Anal.* Calcd for $C_{35}H_{52}N_4O_9 \cdot 2H_2O$: C, 59.30; H, 7.96; N, 7.90. Found: C, 59.19; H, 7.56; N, 7.65. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.75 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 5.22 (1H, brs, $W_{1/2}$=8 Hz, 3α-H), 6.30 (1H, d, J=9.8 Hz, 23-H), 7.34 (1H, d, J=2.6 Hz, 21-H), 7.97 (1H, dd, J=9.8, 2.6 Hz, 22-H).

Assay for the Inhibition of Na^+,K^+-ATPase——The samples were tested for inhibitory activity towards Na^+,K^+-ATPase (10 μmol Pi/mg protein) from guinea pig heart by the method described in the previous paper.[3] The concentration of a compound required for 50% inhibition was defined as the I_{50} value.

Acknowledgement The authors thank the staff of the central analytical laboratory of this institute for elemental analyses and spectral measurements. This work was supported in part by a grant from the Ministry of Education, Science and Culture.

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10) K. Shimada, M. Hasegawa, K. Hasebe, Y. Fujii, and T. Nambara, *Chem. Pharm. Bull.*, **24**, 2995 (1976).
11) The I_{50} value of this compound reported in the previous paper[2] should be corrected.

[Chem. Pharm. Bull. 35(6)2305—2313(1987)]

Asymmetric Synthesis Using Chiral Acetals: Studies on the Nucleophilic Addition of Organometallics to Chiral α-Keto Acetals in Cyclic Systems

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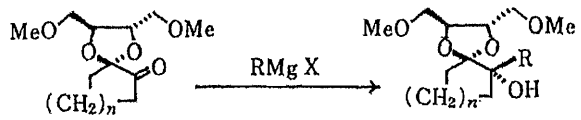
Four chiral cyclic α-keto acetals (**3a**—**d**) were prepared through transacetalization of the α-hydroxydimethyl acetals [**1**($n=1$, $n=2$)] with (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol or (−)-(2*R*,3*R*)-2,3-butanediol and reacted with organometallic reagents (Grignard reagents and organolithium reagents). The reactions of the α-keto acetals (**3a**, **3b**) derived from **1** and (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol with Grignard reagents proceeded in a highly diastereoselective manner.

Keywords——Asymmetric synthesis; diastereoselective nucleophilic addition; chiral cyclic α-keto acetal; (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol; (−)-(2*R*,3*R*)-2,3-butanediol; Grignard reagent; organolithium reagent

Asymmetric reactions using the chiral acetals prepared from chiral diols with C_2 axis of symmetry have recently been receiving much attention from organic chemists. The reactions can be divided broadly into two groups. The first group consists of asymmetric reactions using the chiral acetals as synthetic equivalents of carbonyl compounds as exemplified by studies on asymmetric cyclization,[1a] Lewis acid-catalyzed coupling of chiral acetals with organosilicon compounds,[1b] and reaction of chiral acetals with organometallic compounds.[1c] The second group involves asymmetric reactions on a prochiral center using the neighboring chiral acetals as chiral auxiliaries, as reported in studies on asymmetric amination,[2a] asymmetric bromolactonization,[2b] asymmetric reduction,[2c] asymmetric cyclopropanation,[2d] asymmetric bromination,[2e] and asymmetric Michael addition.[2f] Very recently we have briefly reported[3] the highly diastereoselective nucleophilic addition of Grignard reagents to chiral cyclic α-keto acetals (**3a** and **3b**) prepared from (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol, in which the chiral acetal works as a protective group of the ketone and as the chiral auxiliary at the same time. Here we present a full account of this work on the nucleophilic addition of organometallics to chiral α-keto acetals in cyclic systems.



3a ($n=1$) **3b** ($n=2$) **4aA** **4bA**

Chart 1

Results and Discussion

Two different types of α-keto acetals, **3a** and **3b** derived from (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol,[4] and **3c** and **3d** derived from (−)-(2*R*,3*R*)-2,3-butanediol,[5] were prepared and used to examine nucleophilic addition in cyclic systems.

Syntheses of Chiral α-Keto Acetals (3a—d)

The α-keto acetals (**3a—d**) were readily prepared from the corresponding α-hydroxydimethyl acetals (**1**)[6] as shown in Chart 2. Transacetalization of α-hydroxydimethyl acetals (**1**) with 1.2 mol eq of the chiral diols [(−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol or (−)-(2*R*,3*R*)-2,3-butanediol] in the presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH) in dichloromethane (CH_2Cl_2) afforded α-hydroxy acetals (**2a—d**) as an epimeric mixture at the secondary alcohol moiety. Oxidation of the α-hydroxy acetals (**2a—d**) to the corresponding α-keto acetals (**3a—d**) was achieved by modified pyridinium dichlorochromate (PDC) oxidation [PDC/acetic anhydride (Ac_2O)/molecular sieves 3A/CH_2Cl_2; although acetic acid (AcOH) was used in the literature,[7] **2a—d** could be oxidized more rapidly by using Ac_2O in place of AcOH] after several trials with pyridinium chlorochromate (PCC),[8] PDC,[9] and PCC–alumina.[10]

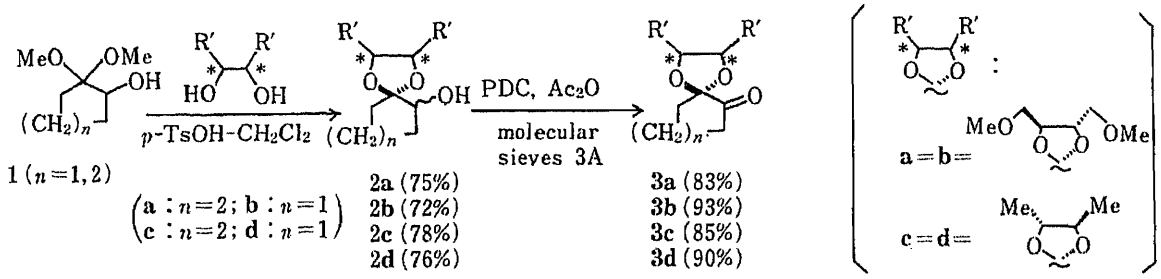


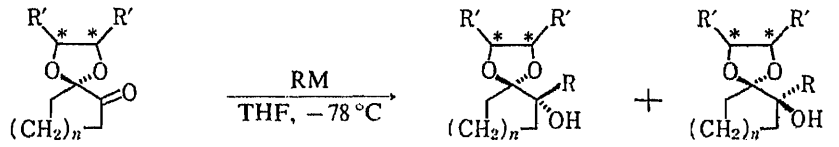
Chart 2

Nucleophilic Addition of Organometallics to α-Keto Acetals (3a—d)

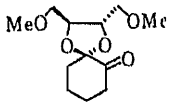
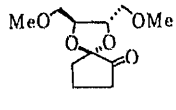
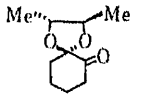
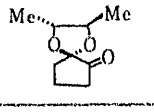
The reactions of the α-keto acetals (**3a—d**) with 5 mol eq of organometallic reagents are summarized in Table I. In the reactions of **3a—d** with methylmagnesium bromide (MeMgBr) or methyllithium (MeLi) in tetrahydrofuran (THF) at −78 °C (runs 1, 2, 11, 12, 19—22), extremely high stereoselectivity was observed in the reactions of **3a** and **3b** with MeMgBr (runs 1, 11). However, relatively poor selectivity was obtained in the reactions of **3a** and **3b** with MeLi (runs 2, 12) and in the reactions of **3c** and **3d** with MeMgBr and MeLi (runs 19—22). In the stereoselective reaction of **3a** and **3b** with MeMgBr, THF is a slightly more effective solvent than ether (runs 1, 3, 11, 13) and a lower reaction temperature gives a better outcome (runs 1, 4, 5, 11, 14, 15). The reactions of **3a** and **3b** with other Grignard reagents in THF at −78 °C also gave high stereoselectivity (runs 6—8, 16, 17).

The stereochemistry of the products was determined as follows. The product from **3a** in run 1 was treated with 80% CF_3COOH to afford 2-hydroxy-2-methylcyclohexanone (**5**), whose specific rotation [$[\alpha]_D$ +100° (c=0.2, $CHCl_3$)] showed good agreement with the reported value (+100.5°).[11] This result was confirmed by applying the Horeau's method[12] to the diols (**6** and **7**) obtained by reduction of **5**. Thus, the absolute configurations of the secondary alcohols of **6** and **7** were assigned as *S* for **6** and *R* for **7** from the specific rotation of the recovered α-phenylbutyric acid. Since the relative stereochemistry of **6** and **7** was determined to be *cis* for **6** and *trans* for **7** by comparison with racemic **6** obtained by osmium tetroxide (OsO_4) oxidation of 1-methylcyclohexene (**10**), the stereochemistry of the tertiary alcohol of **4a**A (R = Me) was deduced (Chart 3). The stereochemistries of the products in runs

TABLE I. Nucleophilic Addition of RM to **3a—d**



3a—3d **4aA—4dA** **4aB—4dB**

Run	Substrate		RM	Yield (%)	Ratio (A : B)
1		**3a**	MeMgBr	93	100 : 0[d]
2		**3a**	MeLi	85	40 : 60[d]
3		**3a**	MeMgBr[a]	86	92 : 8[d]
4		**3a**	MeMgBr[b]	94	99 : 1[d]
5		**3a**	MeMgBr[c]	93	96 : 4[d]
6		**3a**	EtMgCl	95	100 : 0[e]
7		**3a**		95	97 : 3[e]
8		**3a**	PhMgBr	85	95 : 5[f]
9		**3a**		79	30 : 70[e]
10		**3a**	PhLi	75	30 : 70[f]
11		**3b**	MeMgBr	91	98 : 2[f]
12		**3b**	MeLi	83	50 : 50[f]
13		**3b**	MeMgBr[a]	91	97 : 3[f]
14		**3b**	MeMgBr[b]	90	95 : 5[f]
15		**3b**	MeMgBr[c]	86	94 : 6[f]
16		**3b**	EtMgCl	95	100 : 0[e]
17		**3b**		95	97 : 3[f]
18		**3b**		60	60 : 40[f]
19		**3c**	MeMgBr	93	60 : 40[d]
20		**3c**	MeLi	96	65 : 35[d]
21		**3d**	MeMgBr	95	70 : 30[f]
22		**3d**	MeLi	90	40 : 60[f]

a) The reaction was carried out in ether at −78 °C. *b*) The reaction was carried out at 0 °C. *c*) The reaction was carried out under reflux. *d*) Determined by ^{1}H-NMR spectroscopy from the ratios of the singlet signals due to the tertiary methyl protons. *e*) Determined by ^{1}H-NMR spectroscopy from the ratios of the singlet signals due to the methoxymethyl protons. *f*) Determined by GLC analysis.

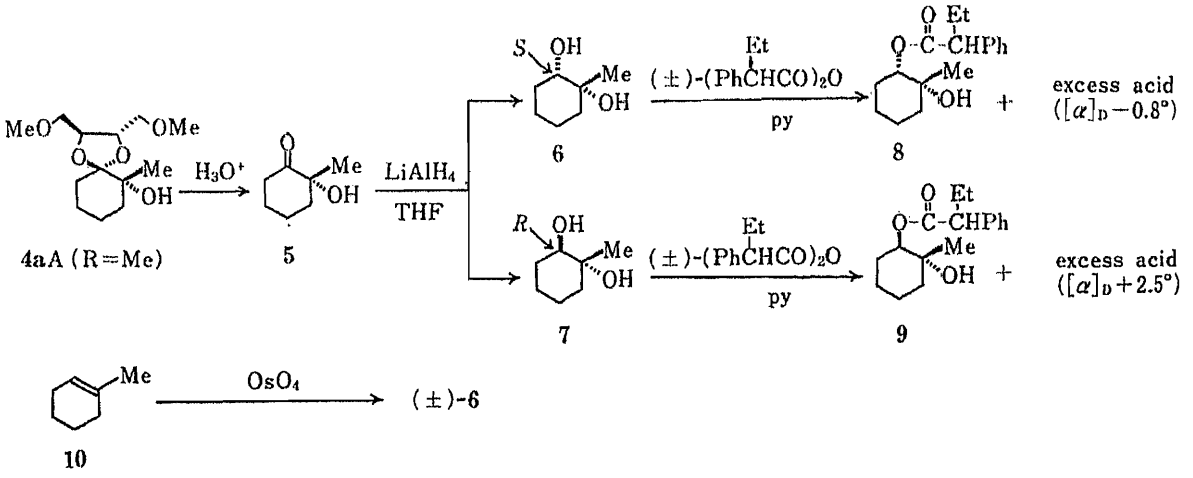


Chart 3

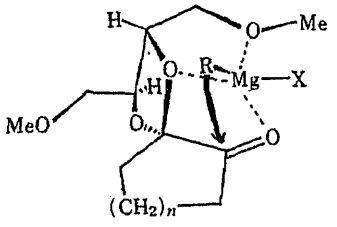


Fig. 1

6, 8, 10, 11—16, and 19—22 were also determined by comparing the specific rotation of the corresponding α-hydroxy-α-substituted cycloalkanones obtained by acid hydrolysis of the products in runs 6, 8, 11, 16, 19, and 21 with the reported values.[11] The stereochemistries of the products in runs 7, 9, 17, and 18 were determined by correlation to the products in runs 6 and 16 after hydrogenation (H_2/5%Pd–C) (see Experimental).

The attainment of extremely high stereoselectivity in the reactions of **3a** and **3b** with Grignard reagents might be rationalized as follows. That is, the formation of the predominant products in the reactions of **3a** and **3b** with Grignard reagents can be explained by the attack of the reagents on the *si*-face of the ketones. This result would suggest chelation of the magnesium metal of the reagents between the carbonyl oxygen atom, the methoxy oxygen atom, and one of the acetal oxygen atoms. Although the conformation of **3a** in the transition state of the reaction is still not clear, this consideration seems valid in the case of **3b** because the conformation of **3b** is fixed as illustrated in Fig. 1. The reason for the poor stereoselectivity in the reactions of **3a** and **3b** with organolithium reagents is still not clear.[13]

As mentioned above, (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol proved to be a desirable chiral auxiliary in the nucleophilic addition of Grignard reagents to chiral α-keto acetals in cyclic systems.

Experimental

The following instruments were used to obtain physical data: specific rotation, Perkin–Elmer 241 polarimeter; infrared (IR) spectra, JASCO IRA-1 spectrometer; proton nuclear magnetic resonance (^{1}H-NMR) spectra, Hitachi R-22 (90 MHz), JEOL JNM-FX 90Q FT-NMR (90 MHz), or JEOL JNM-GX 500 FT-NMR (500 MHz) spectrometer (with tetramethylsilane as an internal standard); low- and high-resolution mass spectra (MS), JEOL LMS D-300 mass spectrometer (with a direct inlet system). A Shimadzu GC-4B gas chromatograph was used for gas liquid chromatographic (GLC) analysis (PEG-20M 15 m glass capillary column, with nitrogen as a carrier gas). E. Merck silica-gel (0.063—0.200 mm, 70—230 mesh ASTM) for column chromatography and E. Merck TLC plates pre-coated with Silica gel $60F_{254}$ for preparative thin-layer chromatography (TLC) (0.5 mm) and TLC detection (0.2 mm) were used. Specific rotation was measured at 20 °C in $CHCl_3$, unless otherwise mentioned. All melting points are uncorrected.

Syntheses of α-Hydroxy Acetals (2a—d)——General Procedure: A catalytic amount of *p*-TsOH was added to a mixture of the α-hydroxydimethyl acetal (**1**) (4.1 mmol) and (−)-(2*S*,3*S*)-1,4-dimethoxy-2,3-butanediol (**21**) (4.9 mmol) [or (−)-(2*R*,3*R*)-2,3-butanediol (**22**) (4.9 mmol)] in anhydrous CH_2Cl_2 (12 ml), and the resulting solution was stirred for 12 h at room temperature under a nitrogen atmosphere. Then K_2CO_3 (one microspatula-full) was added with stirring and after 10 min the precipitates were filtered off. The filtrate was concentrated under reduced pressure. The residue was purified on an SiO_2 column chromatography to give **2** as a diastereomeric mixture.

The α-hydroxy acetal **2a** (756 mg, 75%) was prepared from **1** (n=2) (655 mg) and **21** (735 mg) (eluent, hexane : ether = 1 : 3). Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3445, 1100. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.2—1.9 (8H, m, $-CH_2-\times4$), 3.39 (3/2H, s, $-OCH_3\times1/2$), 3.40 (3/2H, s, $-OCH_3\times1/2$), 3.42 (3H, s, $-OCH_3$), 3.5—3.7 (5H, m, $-CH_2OCH_3\times2$ and $-HCOH-$), 3.98 (1H center, m, $-O-CH-$), 4.30 (1H center, m, $-O-CH-$). Exact mass Calcd for $C_{12}H_{22}O_5$: 246.1465. Found: 246.1460.

The α-hydroxy acetal **2b** (686 mg, 72%) was prepared from **1** (n=1) (600 mg) and **21** (735 mg) (eluent, hexane : ether = 1 : 3). Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3455, 1100. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.5—2.0 (6H, m, $-CH_2-\times3$), 3.40 (6H, s, $-OCH_3\times2$), 3.4—3.6 (4H, m, $-CH_2OCH_3\times2$), 3.6—4.2 (3H, m, $-O-CH-\times2$ and $-HCOH$). Exact mass Calcd for $C_{11}H_{20}O_5$: 232.1308. Found: 232.1285.

The α-hydroxy acetal **2c** (594 mg, 78%) was prepared from **1** (n=2) (655 mg) and **22** (440 mg) (eluent, hexane : ether = 1 : 1). Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1095. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.2—1.35 (6H,

m, -CHCH$_3$×2), 1.4—2.0 (8H, m, -CH$_2$-×4), 3.4—3.8 (3H, m, -O-CHCH$_3$×2 and -HCOH). Exact mass Calcd for $C_{10}H_{18}O_3$: 186.1273. Found: 186.1257.

The α-hydroxy acetal **2d** (537 mg, 76%) was prepared from **1** ($n=1$, 600 mg) and **22** (440 mg) (eluent, hexane : ether = 5 : 1). Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3565, 1110. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.1—1.3 (6H, m, -CHCH$_3$×2), 1.4—2.0 (6H, m, -CH$_2$-×3), 2.2 (1H, brs, -OH), 3.4—3.8 (3H, m, -CH-CH$_3$×2 and -HCOH). Exact mass Calcd for $C_9H_{16}O_3$: 172.1096. Found: 172.1081.

Syntheses of α-Keto Acetals (3a—d)——General Procedure: Activated molecular sieves 3A (2.19 g), PDC (4.1 mmol), and Ac_2O (0.27 ml) were added to a solution of **2** (2.7 mmol) in anhydrous CH_2Cl_2 (4 ml) at 0 °C, and the resulting mixture was stirred for 1 h at room temperature under a nitrogen atmosphere. The precipitates were removed by passage through a short Celite column [ether then ethyl acetate (EtOAc)]. The filtrate was concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel to give **3**.

1,2-Cyclohexanedione mono-1,4-di-*O*-methyl-L-threitol acetal **3a** (547 mg) was prepared from **2a** (664 mg) in 83% yield (eluent, hexane : ether = 2 : 1). **3a**: Colorless oil, $[\alpha]_D$ −29° ($c=2.6$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1100. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.6—2.0 (6H, m, -CH$_2$-×3), 2.4—2.7 (2H, m, -CH$_2$-), 3.37, 3.40 (3H each, both s, -OCH$_3$×2), 3.4—3.6 (4H, m, -CH$_2$-OCH$_3$×2), 3.9—4.1 (2H, m, -O-CH-×2). *Anal.* Calcd for $C_{12}H_{20}O_5$: C, 59.00; H, 8.25. Found: C, 58.84; H, 8.44.

1,2-Cyclopentanedione mono-1,4-di-*O*-methyl-L-threitol acetal **3b** (583 mg) was obtained from **2b** (632 mg) in 93% yield (eluent, hexane : ether = 1 : 1). **3b**: Colorless oil, $[\alpha]_D$ −19° ($c=0.7$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1095. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.7—2.4 (6H, m, -CH$_2$-×3), 3.40 (6H, s, -OCH$_3$×2), 3.5—3.8 (4H, m, -CH$_2$OCH$_3$×2), 3.9—4.4 (2H, m, -O-CH-×2). *Anal.* Calcd for $C_{11}H_{18}O_5$: C, 57.38; H, 7.88. Found: C, 57.40; H, 8.03.

1,2-Cyclohexanedione mono-(2*R*,3*R*)-(−)-2,3-butanediol acetal **3c** (422 mg) was obtained from **2c** (502 mg) in 85% yield (eluent, hexane : ether = 3 : 2). **3c**: Colorless oil, $[\alpha]_D$ +18.2° (c = 0.86). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1085. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.23 (3H, d, $J=5.7$ Hz, -CH-CH$_3$), 1.28 (3H, d, $J=5.7$ Hz, -CH-CH$_3$), 1.6—2.0 (6H, m, -CH$_2$-×3), 2.4—2.7 (2H, m, -CH$_2$-), 3.4—3.9 (2H, m, -O-CH-×2). Exact mass Calcd for $C_{10}H_{16}O_3$: 184.1100. Found: 184.1107.

1,2-Cyclopentanedione mono-(2*R*,3*R*)-(−)-2,3-butanediol acetal **3d** (413 mg) was obtained from **2d** (464 mg) in 90% yield (eluent, hexane : ether = 4 : 1). **3d**: Colorless oil, $[\alpha]_D$ +1.3° ($c=1.5$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1075. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.26 (3H, d, $J=6.2$ Hz, -CH-CH$_3$), 1.32 (3H, d, $J=5.9$ Hz, -CH-CH$_3$), 1.9—2.1 (4H, m, -CH$_2$-×2), 2.2—2.4 (2H, m, -CH$_2$-), 3.67 (1H, A in ABX_3, $J_{AB}=8.1$ Hz, $J_{AX}=5.9$ Hz, -CH-CH$_3$), 4.04 (1H, B in ABX_3, $J_{AB}=8.1$ Hz, $J_{BX}=6.2$ Hz, -CH-CH$_3$). Exact mass Calcd for $C_9H_{14}O_3$: 170.0943. Found: 170.0958.

Nucleophilic Addition of Organometallics to α-Keto Acetals (3a—d)——General Procedure: An organometallic reagent (Grignard reagent or organolithium reagent, 5 mmol, 1—1.5 M solution) was added dropwise to a stirred solution of **3** (1 mmol) in anhydrous THF (10 ml), and the resulting mixture was stirred for 2 h at −78 °C under a nitrogen atmosphere. The excess reagent was decomposed with aq. sat. NH_4Cl at −78 °C and then the mixture was allowed to warm to room temperature. The resulting mixture was extracted with ether. The organic layer was washed with aq. sat. NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel using hexane–ether as an eluting solvent to give **4**.

Run 1: The product [**4a**A (R = Me), 46 mg] was prepared from **3a** (46 mg, 0.19 mmol) and MeMgBr in 93% yield. **4a**A (R = Me): Colorless oil, $[\alpha]_D$ −10.5° ($c=2.2$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3450, 1090. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.31 (3H, s, -C(OH)-CH$_3$), 1.0—2.1 (8H, m, -CH$_2$ ×4), 3.04, 3.09 (3H each, both s, -OCH$_3$×2), 3.2—3.4 (4H, m, -CH$_2$-OCH$_3$×2) 3.9—4.2 (2H, m, -O-CH-×2). Exact mass Calcd for $C_{13}H_{24}O_5$: 260.1621. Found: 260.1591.

Run 2: The product [**4a**A (R = Me): **4a**B (R = Me) = 40 : 60, 11 mg] was prepared from **3a** (12 mg, 0.05 mmol) and MeLi in 85% yield. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3450, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.31, 1.40 (total 3H, both s, ratio 2 : 3, -C(OH)-CH$_3$), 1.4—2.0 (8H, m, -CH$_2$-×4), 3.00, 3.04, 3.07, 3.09 (total 6H, all s, ratio 3 : 2 : 3 : 2, -OCH$_3$×2), 3.2—3.5 (4H, m, -CH$_2$-OCH$_3$×2), 3.8—4.5 (2H, m, -O-CH-×2). Exact mass Calcd for $C_{13}H_{24}O_5$: 260.1621. Found: 260.1642.

Run 3—5: The products were prepared from **3a** and MeMgBr under the conditions shown in Table 1. The ratios of the products were determined by ^{1}H-NMR (see run 2).

Run 6: The product [**4a**A (R = Et), 57 mg] was prepared from **3a** (57 mg, 0.23 mmol) and EtMgCl in 95% yield. **4a**A (R = Et): Colorless oil, $[\alpha]_D$ −19° ($c=1.2$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3580, 3450, 1090. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.01 (3H, t, $J=7.5$ Hz, -CH$_2$CH$_3$), 1.2—2.1 (10H, m, -CH$_2$-CH$_3$ and -CH$_2$-×4), 3.07, 3.11 (3H each, both s, -OCH$_3$×2), 3.9—4.1 (2H, m, -O-CH-×2). *Anal.* Calcd for $C_{14}H_{26}O_5$: C, 61.29; H, 9.55. Found: C, 61.13; H, 9.85.

Run 7: The product [**4a**A (R = vinyl), 94% diastereomeric excess (de), 18 mg] was prepared from **3a** (18 mg, 0.07 mmol) and vinylmagnesium bromide in 95% yield. **4a**A (R = vinyl, 94% de): Colorless oil, $[\alpha]_D$ −44.0° ($c=0.85$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3425, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.3—2.8 (8H, m, -CH$_2$-×4), 3.09, 3.11 (3H each, both s, -OCH$_3$×2), 3.31—3.45 (4H, m, -CH$_2$-×2), 3.80—4.30 (2H, m, -O-CH-×2), 5.21 (1H, dd, $J=2$, 11 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 5.54 (1H, dd, $J=2$, 17 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 6.48 (1H, dd, $J=11$, 17 Hz, -CH=CH$_2$). *Anal.* Calcd for $C_{14}H_{24}O_5$: C, 61.74; H, 8.88. Found: C, 61.71; H, 8.99.

Run 8: The product [**4a**A (R = Ph), 90% de, 36 mg] was prepared from **3a** (32 mg, 0.13 mmol) and PhMgBr in 85% yield (GLC analysis under the same conditions as for run 10). **4a**A (R = Ph, 90% de): Colorless oil, $[\alpha]_D$ −34.1° (c = 2.0). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3450, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.3—2.0 (6H, m, $-CH_2-\times 3$), 2.0—2.4 (2H, m, $-CH_2-$), 2.98, 3.03 (3H each, both s, $-OCH_3\times 2$), 2.7—3.2 (5H, m, $-CH_2-OCH_3\times 2$ and $-O-CH-$), 3.8—4.1 (1H, m, $-O-CH-$), 7.0—7.3, 7.5—7.8 (5H, m, aromatic protons). *Anal.* Calcd for $C_{18}H_{26}O_5$: C, 67.06; H, 8.13. Found: C, 66.94; H, 8.32.

Run 9: The product [**4a**A (R = vinyl): **4a**B (R = vinyl) = 30 : 70, 31 mg] was prepared from **3a** (36 mg, 0.15 mmol) and vinyllithium in 79% yield. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2935, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.3—2.8 (8H, m, $-CH_2-\times 4$), 3.08, 3.09, 3.11, 3.12 (total 6H, all s, ratio 7 : 3 : 3 : 7, $-OCH_3\times 2$), 3.31—3.45 (4H, m, $-CH_2-\times 2$), 3.80—4.30 (2H, m, $-O-CH-\times 2$), 5.21, 5.24 (total 1H, each dd, ratio 3 : 7, J = 2, 11 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 5.54, 5.63 (total 1H, each dd, ratio 3 : 7, J = 2, 17 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 6.29, 6.48 (total 1H, each dd, ratio 7 : 3, J = 11, 17 Hz, $-CH=CH_2$). Exact mass Calcd for $C_{14}H_{24}O_5$: 272.1621. Found: 272.1591.

Run 10: The product [**4a**A (R = Ph): **4a**B (R = Ph) = 30 : 70, 23.7 mg] was prepared from **3a** (24 mg, 0.1 mmol) and PhLi in 75% yield [GLC analysis: column temp. 130 °C; flow rate 1 ml/min; retention time (t_R), **4a**A (R = Ph), 140.5 min, **4a**B (R = Ph) 120.1 min]. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2930, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.2—2.0 (6H, m, $-CH_2-\times 3$), 1.3—2.0 (2H, m, $-CH_2-$), 2.7—3.2 (5H, m, $-CH_2-OCH_3\times 2$ and $-O-CH-$), 2.94, 2.98, 3.03 (total 6H, all s, ratio 7 : 3 : 10, $-OCH_3\times 2$), 3.8—4.1 (1H, m, $-O-CH-$), 7.0—7.3, 7.5—7.8 (5H, m, aromatic protons). Exact mass Calcd for $C_{18}H_{26}O_5$: 322.1777. Found: 322.1767.

Run 11: The product [**4b**A (R = Me), 96% de, 29 mg] was prepared from **3b** (30 mg, 0.13 mmol) and MeMgBr in 91% yield (GLC analysis under the same conditions as for run 12). **4b**A (R = Me, 96% de): Colorless oil, $[\alpha]_D$ −9.9° (c = 0.99). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3580, 3450, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.32 (3H, s, $-C(OH)-CH_3$), 1.4—2.4 (6H, m, $-CH_2-\times 3$), 3.05, 3.08 (3H each, both s, $-OCH_3\times 2$), 3.2—3.4 (4H, m, $-CH_2-OCH_3\times 2$), 3.9—4.1 (2H, m, $-O-CH-\times 2$). Exact mass Calcd for $C_{12}H_{22}O_5$: 246.1468. Found: 246.1474.

Run 12: The product [**4b**A (R = Me): **4b**B (R = Me) = 50 : 50, 12.4 mg] was prepared from **3b** (14 mg, 0.06 mmol) and MeLi in 83% yield [GLC analysis: column temp. 140 °C; flow rate 1 ml/min; t_R, **4b**A (R = Me), 34.8 min, **4b**B (R = Me), 31.3 min]. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.32, 1.39 (total 3H, each s, ratio 1 : 1, $-C(OH)-CH_3$), 1.4—2.4 (6H, m, $-CH_2-\times 3$), 2.99, 3.05, 3.08 (total 6H, all s, ratio 1 : 2 : 1, $-OCH_3\times 2$), 3.2—3.4 (4H, m, $-CH_2-OCH_3\times 2$), 3.8—4.4 (2H, m, $-O-CH-\times 2$). Exact mass Calcd for $C_{12}H_{22}O_5$: 246.1468. Found: 246.1484.

Runs 13—15: The products were prepared from **3b** and MeMgBr under the conditions shown in Table I. The ratios of the products were determined by GLC analysis as described for run 12.

Run 16: The product [**4b**A (R = Et), 94 mg] was prepared from **3b** (87 mg, 0.38 mmol) and EtMgCl in 95% yield. **4b**A (R = Et): Colorless oil, $[\alpha]_D$ 22.4° (c = 1.59). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3450, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.07 (3H, t, J = 7.3 Hz, $-CH_2CH_3$), 1.4—2.0 (6H, m, $-CH_2CH_3$ and $-CH_2-\times 2$), 2.0—2.3 (2H, m, $-CH_2-$), 3.09, 3.11 (3H each, both s, $-OCH_3\times 2$), 3.33 (2H, d, J = 6.4 Hz, $-CH_2-OCH_3$), 3.38 (2H, d, J = 6.3 Hz, $-CH_2-OCH_3$), 3.8—4.2 (2H, m, $-O-CH-\times 2$). *Anal.* Calcd for $C_{13}H_{24}O_5$: C, 59.98; H, 9.29. Found: C, 59.93; H, 9.53.

Run 17: The product [**4b**A (R = vinyl), 94% de, 24 mg] was prepared from **3b** (22 mg, 0.096 mmol) and vinylmagnesium bromide in 95% yield [GLC analysis: column temp. 125 °C; flow rate 1 ml/min; t_R, **4b**A (R = vinyl), 11.5 min, **4b**B (R = vinyl), 9.9 min]. **4b**A (R = vinyl, 94% de): Colorless oil, $[\alpha]_D$ −73.4° (c = 0.18). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3540, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.5—2.1 (4H, m, $-CH_2-\times 2$), 2.2—2.4 (2H, m, $-CH_2-$), 3.07, 3.08 (3H each, both s, $-OCH_3\times 2$), 3.2—3.4 (4H, m, $-CH_2-OCH_3\times 2$), 3.9—4.1 (2H, m, $-O-CH-\times 2$), 5.18 (1H, dd, J = 2.2, 10.6 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 5.54 (1H, dd, J = 2.2, 18.0 Hz, $^{H}\!>\!C=C\!<^{H}_{H}$), 6.36 (1H, dd, J = 10.6, 18.0 Hz, $-CH=CH_2$). *Anal.* Cacd for $C_{13}H_{22}O_5$: C, 60.44; H, 8.59. Found: C, 60.56; H, 8.87.

Run 18: The product [**4b**A (R = vinyl): **4b**B (R = vinyl) = 60 : 40, 35 mg] was prepared from **3b** (32 mg, 0.23 mmol) and vinyllithium in 60% yield. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3540, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.5—2.1 (4H, m, $-CH_2-\times 2$), 2.2—2.4 (2H, m, $-CH_2-$), 3.03, 3.04, 3.07, 3.08 (total 6H, each s, ratio 4 : 4 : 6 : 6, $-OCH_3\times 2$), 3.2—3.4 (4H, m, $-CH_2-OCH_3\times 2$), 3.8—4.1 (2H, m, $-O-CH-\times 2$), 5.17, 5.18 (total 1H, each dd, J = 2.2, 10.6 Hz, ratio 4 : 6, $^{H}\!>\!C=C\!<^{H}_{H}$), 5.54, 5.58 (total 1H, each dd, J = 2.2, 18.0 Hz, ratio 6 : 4, $^{H}\!>\!C=C\!<^{H}_{H}$), 6.20, 6.36 (total 1H, each dd, J = 10.6, 18.0 Hz, ratio 4 : 6, $-CH=CH_2$). Exact mass Calcd for $C_{13}H_{22}O_5$: 258.1465. Found: 258.1452.

Run 19: The product [**4c**A (R = Me): **4c**B (R = Me) = 60 : 40, 69 mg] was prepared from **3c** (69 mg, 0.38 mmol) and MeMgBr in 95% yield. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3580, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 0.92—1.06 (total 6H, m, $-CH-CH_3\times 2$), 1.23, 1.29 (total 3H, each s, ratio 4 : 6, $-C(OH)-CH_3$), 1.4—2.2 (8H, m, $-CH_2-\times 4$), 3.2—3.7 (2H, m, $-O-CH-CH_3\times 2$). Exact mass Calcd for $C_{11}H_{20}O_3$: 200.1413. Found: 200.1425.

Run 20: The product [**4c**A (R = Me): **4c**B (R = Me) = 65 : 35, 20 mg] was prepared from **3c** (19 mg, 0.1 mmol) and MeLi in 96% yield.

Run 21: The product [**4d**A (R = Me): **4d**B (R = Me) = 70 : 30, 17 mg] was prepared from **3d** (16 mg, 0.1 mmol) and MeMgBr in 95% yield [GLC analysis: column temp. 45°C; flow rate 1 ml/min; t_R, **4d**A, 19.2 min, **4d**B, 23.1 min]. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3580, 2980, 1140, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 0.8—1.0 (total 6H, m, -CH-CH$_3$ × 2), 1.29, 1.32 (total 3H, each s, ratio 7 : 3, -C(OH)-CH$_3$), 1.4—2.4 (6H, m, -CH$_2$- × 3), 3.1—3.6 (2H, m, -O-CH-CH$_3$ × 2). Exact mass Calcd for $C_{10}H_{18}O_3$: 186.1256. Found: 186.1258.

Run 22: The product [**4d**A (R = Me): **4d**B (R = Me) = 40 : 60, 20 mg] was prepared from **3d** (20 mg, 0.12 mmol) and MeLi in 90% yield.

Acid Hydrolysis (Syntheses of α-Hydroxy-α-Substituted Cycloalkanones)——General Procedure: The products obtained in runs 1, 6, 8, 11, 16, 19, and 21 were converted into the corresponding α-hydroxy-α-substituted cycloalkanones as follows. Each product (0.15 mmol) was treated with aq. 80% CF_3COOH (3 ml) at 0°C. The reaction mixture was stirred for 3 h at room temperature, then treated with aq. sat. $NaHCO_3$ at 0°C until it became alkaline. The resulting mixture was extracted with CH_2Cl_2 (20 ml, twice). The organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified on an SiO_2 column using hexane–ether as an eluent to afford the 2-hydroxy-2-alkylcycloalkanone.

(*R*)-(+)-2-Hydroxy-2-methylcyclohexanone (**5**) (36 mg, 64%) was prepared from the product (115 mg) in run 1: colorless oil, $[\alpha]_D$ +100° (*c* = 0.53) (lit.[11] +99.8° as 95% ee). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3475, 1710. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.8—1.0 (2H, m, -CH$_2$-), 1.1—1.4 (2H, m, -CH$_2$-), 1.40 (3H, s, -CH$_3$), 1.5—2.0 (2H, m, -CH$_2$-), 2.3—2.6 (2H, m, -CH$_2$-). Exact mass Calcd for $C_7H_{12}O_2$: 128.0837. Found: 128.0854.

(*R*)-(+)-2-Hydroxy-2-ethylcyclohexanone (15 mg, 70%) was prepared from the product (41 mg) in run 6: colorless oil, $[\alpha]_D$ +134° (*c* = 0.83) (lit.[11] +131.5° as 92% ee). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3475, 1710. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.81 (3H, t, *J* = 7.4 Hz, -CH$_2$CH$_3$), 1.4—2.0 (6H, m, -CH$_2$- × 3), 2.0—2.6 (4H, m, -CH$_2$- × 2). Exact mass Calcd for $C_8H_{14}O_2$: 142.0994. Found: 142.1000.

(*S*)-(+)-2-Hydroxy-2-phenylcyclohexanone (7 mg, 49%) was prepared from the product (24 mg) in run 8: colorless oil, $[\alpha]_D$ +185° (*c* = 0.12) (lit.[11] +169.3° as 58% ee; slight racemization occurred during acid hydrolysis). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3475, 1710. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.3—1.5 (2H, m, -CH$_2$-), 1.5—2.3 (4H, m, -CH$_2$- × 2), 2.3—2.7 (2H, m, -CH$_2$-), 7.4 (5H, brs, aromatic protons). Exact mass Calcd for $C_{12}H_{14}O_2$: 190.0994. Found: 190.1014.

(*R*)-(+)-2-Hydroxy-2-methylcyclopentanone (11 mg, 40%) was prepared from the product (62 mg) in run 11: colorless oil, $[\alpha]_D$ +27° (*c* = 0.13) (lit.[11] +27.4° as 95% ee). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1740. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.26 (3H, s, -CH$_3$), 1.8—2.7 (6H, m, -CH$_2$- × 3). Exact mass Calcd for $C_6H_{10}O_2$: 114.0681. Found: 114.0698.

(*R*)-(+)-2-Hydroxy-2-ethylcyclopentanone (41 mg, 75%) was prepared from the product (110 mg) in run 16: colorless oil, $[\alpha]_D$ +94° (*c* = 0.16). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1740. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.93 (3H, t, *J* = 7.2 Hz, -CH$_2$CH$_3$), 1.2—2.5 (8H, m, -CH$_2$CH$_3$ and -CH$_2$- × 3). Exact mass Calcd for $C_7H_{12}O_2$: 128.0838. Found: 128.0859.

(*R*)-(+)-2-Hydroxy-2-methylcyclohexanone (12 mg, 63%) was prepared from the product (30 mg) in run 19. $[\alpha]_D$ +19° (*c* = 0.2).

(*R*)-(+)-2-Hydroxy-2-methylcyclopentanone (22 mg, 60%) was prepared from the product (60 mg) in run 21. $[\alpha]_D$ +10° (*c* = 0.3).

Syntheses of Diols (6, 7) from 2-Hydroxy-2-methylcyclohexanone (5)——$LiAlH_4$ (19 mg, 0.5 mmol) was added to a solution of **5** (25 mg, 0.2 mmol) in anhydrous THF (4 ml) at 0°C and the mixture was stirred for 10 min at the same temperature under a nitrogen atmosphere. The reaction mixture was diluted with ether (10 ml) and treated with EtOAc, MeOH, and water. A white solid appeared, and the solution was dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by preparative TLC (hexane : ether = 1 : 4, developed twice) to give **6** (7 mg, 28%) and **7** (12 mg, 47%). **6**: *Rf* value 0.4 (hexane : ether = 1 : 5), colorless oil, $[\alpha]_D$ −0.8° (*c* = 0.4). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3565, 3420, 2940. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.25 (3H, s, -CH$_3$), 1.0—1.9 (8H, m, -CH$_2$- × 4), 3.38 (1H, m, -CH-OH). Exact mass Calcd for $C_7H_{14}O_2$: 130.0991. Found: 130.0984. **7**: *Rf* value 0.2 (hexane : ether = 1 : 5), colorless needles, mp 69—70°C (hexane), $[\alpha]_D$ −1.4° (*c* = 0.9). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3585, 3425, 2940. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.18 (3H, s, -CH$_3$), 1.0—2.0 (8H, m, -CH$_2$- × 4), 3.46 (1H, m, -CH-OH). Exact mass Calcd for $C_7H_{14}O_2$: 130.0991. Found: 130.0998.

Osmium Tetroxide Oxidation of 1-Methylcyclohexene (10)——A solution of **10** (30 mg, 0.312 mmol) and OsO_4 (79.2 mg, 0.312 mmol) in anhydrous THF (2 ml) was stirred overnight at room temperature. The mixture was treated with 2 ml of $NaHSO_3$–H_2O–pyridine (2 g : 30 ml : 35 ml) solution for 1 h. The resulting mixture was extracted with CH_2Cl_2. The organic layer was washed with aq. 10% HCl, dried over $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified on an SiO_2 column (hexane : ether = 1 : 5) to give (±)-**6** (37 mg, 91%), which was identical with authentic **6** on TLC and ^{1}H-NMR comparisons.

α-Phenylbutyration of Diols (6, 7)——General Procedure: (±)-α-Phenylbutyric anhydride (1.0 mmol) was added to a solution of **6** or **7** (0.1 mmol) in pyridine (2 ml), and the mixture was stirred for 24 h at room temperature. The resulting solution was diluted with water (15 ml), stirred for 30 min, and extracted with EtOAc. The EtOAc layer (No. 1) was extracted with aq. sat. $NaHCO_3$. The aqueous layer and the aq. $NaHCO_3$ layer were combined, washed with

EtOAc, acidified with aq. 5% HCl, and extracted with benzene. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified on an SiO_2 column (hexane : ether = 1 : 1) to give α-phenylbutyric acid. The organic layer (No. 1) was washed with aq. 5% HCl, aq. sat. $NaHCO_3$, and brine successively, dried over $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified on an SiO_2 column (hexane : ether = 1 : 1) to give **8** or **9**.

The ester **8** (8.8 mg, 82%) and α-phenylbutyric acid (106 mg) were obtained from **6** (5.1 mg, 0.039 mmol) and (±)-α-phenylbutyric anhydride (121 mg, 0.39 mmol). **8**: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560, 1730. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.87 (3H, s, $-\underline{C}(OH)-CH_3$), 0.91, 0.93 (total 3H, each t, ratio 3 : 1, J = 7.3 Hz, $\underline{CH_3}-CH_2-$), 1.25—1.45 (4H, m, $-\underline{CH_2}-\times 2$), 1.6—1.7 (2H, m, $-\underline{CH_2}-$), 1.8—1.9 (2H, m, $-\underline{CH_2}-$), 2.1—2.2 (2H, m, $-\underline{CH_2}-$), 3.48, 3.50 (total 1H, each t, ratio 3 : 1, J = 8 Hz, $-\underline{H}CPh-$), 4.61 (1H, dd, J = 7, 7.4 Hz, $-\underline{CH}-O-$), 7.2—7.35 (5H, m, aromatic protons). Exact mass Calcd for $C_{17}H_{24}O_3$: 276.1723. Found: 276.1701. Recovered α-phenylbutyric acid: $[\alpha]_D$ −0.8° (c = 5.3, benzene).

The ester **9** (18.8 mg, 85%) and α-phenylbutyric acid (185 mg) were obtained from **7** (10.5 mg, 0.081 mmol) and (±)-α-phenylbutyric anhydride (251 mg, 0.81 mmol). **9**: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560, 1730. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.91 (3H, t, J = 7.4 Hz, $\underline{CH_3}-CH_2-$), 0.98 (3H, s, $-HOC-\underline{CH_3}$), 1.25—1.5 (4H, m, $-\underline{CH_2}-\times 2$), 1.55—1.7 (2H, m, $-\underline{CH_2}-$), 1.75—1.9 (2H, m, $-\underline{CH_2}-$), 2.1—2.2 (2H, m, $-\underline{CH_2}-$), 3.46, 3.48 (total 1H, each t, ratio 3 : 1, J = 8 Hz, $-\underline{H}CPh-$), 4.65 (1H, dd, J = 3.7, 9.2 Hz, $-\underline{CH}-O-$), 7.2—7.35 (5H, m, aromatic protons). Exact mass Calcd for $C_{17}H_{24}O_3$: 276.1726. Found: 276.1751. Recovered α-phenylbutyric acid: $[\alpha]_D$ +2.5° (c = 4.4, benzene).

Hydrogenation of the Products Obtained in Runs 7, 9, 17 and 18——General Procedure: The products (10 mg) from runs 7, 9, 17 and 18 were hydrogenated in EtOAc (1 ml) in the presence of a catalytic amount of 5% Pd–C under atmospheric pressure at room temperature. After the completion of the reaction, the catalyst was removed by filtration. The filtrate was concentrated under reduced pressure. The residue was purified on an SiO_2 column (hexane–ether) to give the ethyl compound in quantitative yield.

Compound **4a**A (R = Et, 94% de) was prepared from the product of run 7. Compound **4a**A (R = Et) obtained here was identical with the product of run 6 (^{1}H-NMR comparison).

Compounds **4a**A (R = Et) and **4a**B (R = Et) as a mixture (3 : 7) were prepared from the product of run 9. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2945, 1095. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.01, 1.09 (total 3H, each t, ratio 3 : 7, J = 7 Hz, $-CH_2-\underline{CH_3}$), 1.25—2.10 (10H, m, $-\underline{CH_2}-CH_3$ and $-\underline{CH_2}-\times 4$), 3.01, 3.04, 3.08, 3.10 (total 6H, all s, ratio 7 : 3 : 7 : 3, $-OCH_2\times 2$), 3.20—3.45 (4H, m, $-\underline{CH_2}OCH_3\times 2$), 3.98, 4.35 (total 2H, each m, ratio 3 : 7, $-O-\underline{CH}-\times 2$). Exact mass Calcd for $C_{14}H_{26}O_5$: 274.1779. Found: 274.1774.

Compound **4b**A (R = Et, 94% de) was prepared from the product of run 17. Compound **4b**A (R = Et) obtained here was identical with the product of run 16 (^{1}H-NMR comparison).

Compounds **4b**A (R = Et) and **4b**B (R = Et) as a mixture (4 : 6) were prepared from the product of run 18. Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575, 3450, 1100. ^{1}H-NMR (10% solution in C_6D_6) δ: 1.07, 1.15 (total 3H, each t, J = 7.3 Hz, ratio 4 : 6, $-CH_2-\underline{CH_3}$), 1.4—2.0 (6H, m, $-\underline{CH_2}CH_3$ and $-\underline{CH_2}-\times 2$), 2.0—2.3 (2H, m, $-\underline{CH_2}-$), 3.01, 3.09, 3.11, 3.13 (total 6H, all s, ratio 4 : 6 : 6 : 4, $-OCH_3\times 2$), 3.20—3.45 (4H, m, $-\underline{CH_2}OCH_3\times 2$), 3.8—4.2 (2H, m, $-O-\underline{CH}-\times 2$). Exact mass Calcd for $C_{13}H_{24}O_5$: 260.1624. Found: 260.1642.

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Marine Terpenes and Terpenoids. III.[1] Isolation and Structures of Two Cembrane Diols from the Soft Coral *Sinularia mayi*

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Nine cembrane-type diterpenes were isolated from the lipid extract of the soft coral, *Sinularia mayi*. Of these, seven α-methylene-γ-lactone derivatives (**1**—**7**) had been previously isolated from various soft corals. The structures of the two new cembrane diols, sinulariol A (**8a**) and sinulariol B (**9**), were determined by means of spectroscopic analyses, and compound **9** was chemically correlated with nephthenol (**11**) whose absolute configuration is known. Compounds **8a** and **9** are plausible precursors to the cembrane lactones found in various soft corals.

Keywords——Coelenterata; alcyonarian; soft coral; *Sinularia mayi*; cembrane diol; sinulariol A; sinulariol B

Cembrane-type diterpenes occur in relatively few terrestrial organisms such as tobacco leaves, pine resins, or termite secretions.[2] In contrast, they are generally the major components of the lipid fraction of marine Coelenterates such as soft corals (alcyonarians) and, to lesser extent, gorgonians.[2] The significance of the occurrence of cembranoids in these benthic animals, often in vast amounts, is not fully understood. At present, they are believed to act as repellents against predators and in some cases, ichthyotoxic and cytotoxic activities have been reported.[3] Most of the cembranoids found in soft corals have an α-methylene-γ-lactone ring.[3] We report here that the soft coral, *Sinularia mayi*, a common species in coral reefs in southern Japan, contains a variety of cembrane lactones and two new cembrane diols. Cembrane components of *S. mayi* were previously studied by Uchio *et al.* and two hydrocarbons (cembrene and cembrenene), one monooxycembratetraene (mayol) and two simple α-methylene-γ-lactone derivatives were isolated.[4]

Partially dried material of *S. mayi*, collected in the coral reef of Ishigaki island, Okinawa, was extracted with methanol–chloroform and the lipid obtained was subjected to repeated chromatography to give nine cembrane-type diterpenes (**1**—**9**, Chart 1). Seven of them (**1**—**7**) were found to be α-methylene-γ-lactone derivatives that had been reported previously in other species of soft corals.

Compound **1a**, mp 122—123 °C, $[\alpha]_D$ −18°, $C_{22}H_{30}O_6$, was a highly oxidized compound. Analyses of its infrared (IR) spectrum and proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra showed the presence of an epidioxy ring, a *cis*-fused α-methylene-γ-lactone ring, two trisubstituted double bonds and one secondary acetoxyl group. An ^{1}H-NMR decoupling experiment indicated the locations of the lactone ring at C-1 and C-2, and double bonds at C-3 and C-12. The ^{1}H- and ^{13}C-NMR spectra also indicated that the epidioxy oxygens were linked at C-8 and at C-5 or C-11. Selective hydrolysis of the acetoxyl group followed by Jones' oxidation gave an unconjugated ketone **1c**. Recently Uchio *et al.* isolated a novel ichthyotoxic peroxide-containing cembranolide, denticulatolide, from the soft coral *Lobophytum denticulatum* and determined the structure by X-ray crystallography.[5] Direct comparison of the ^{1}H-NMR spectra showed that compound **1a** was identical with

denticulatolide. This compound is of particular interest since it is the first example of a natural cembranoid having an epidioxy moiety in a 14 membered ring. Its biogenesis is unknown, but the presence of the uncommon 12-*cis* double bond suggests a pathway similar to that found in the biosynthesis of prostaglandins.[6] Namely, formation of a radical at C-13 of the ordinary cembrane skeleton followed by combination of two oxygen molecules would lead to the basic structure of **1** (Chart 2). Compound **2** was also isolated by Uchio *et al.*[7] from *L. denticulatum* and the identity of their product with ours was confirmed by comparison of the ^{1}H-NMR spectra.

Compounds **3**—**7** were also known compounds isolated previously from Australian soft coral *Lobophytum* sp. (**3**, **4**, **5**)[8] and from *L. pauciflorum* (**4**, **6**, **7**).[9] Their spectral properties showed good agreement with those in the literature. The structures of compounds **6** and **7** were confirmed by direct comparison of the ^{1}H-NMR spectra with those of authentic samples. The relative stereochemistry of the lactone rings in compounds **2** to **7** was shown to be *cis* as indicated by the large coupling constants between the C-1 and C-2 protons (6.5—8.7 Hz) as compared with those of *trans*-fused lactones (3.5 Hz).[4a]

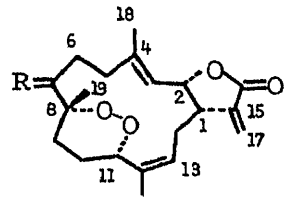
Sinulariol A, mp 116—117.5 °C, $[\alpha]_D$ +101°, $C_{20}H_{32}O_2$, showed hydroxyl (3300 cm^{-1}) and terminal methylene group (1660, 900 cm^{-1}) absorptions in the IR spectrum. Its ^{1}H-NMR spectrum showed signals due to three trisubstituted olefins having a methyl group (δ 1.61, 1.58, 1.55 each 3H, s; 5.43, 1H, br d, J=8.8 Hz; 5.03, 1H, m; 5.00, 1H, m), one tertiary hydroxymethyl (4.07 and 4.17, each 1H, d, J=12.7 Hz), one terminal methylene (4.95 and 5.16, each 1H, s), and one hydroxymethine (4.50, dd, J=8.8, 1.5 Hz). Signals due to doubly allylic methylene protons were absent. If we assume the locations of three double bonds, on biosynthetic grounds, to be at C-3, C-7 and C-11, then the position of one hydroxymethine group should be at C-2, adjacent to the C-3 proton (δ 5.43). A hydroxymethyl group was also assigned at C-16 in view of the absence of an isopropenyl group. The vinylic methyl signals of the isopropenyl side chain in cembrane hydrocarbons occur at slightly lower field (*ca.* 1.75 ppm) than those linked to the 14-membered ring.[3] These facts indicated that sinulariol A corresponds to the diol **8a** (mp 105—106°, $[\alpha]_D$ +82.9°)[4a] synthesized by Uchio *et al.* by reduction of the *cis*-lactone **10** which they previously isolated from *S. mayi*. Direct comparison of the ^{1}H-NMR spectra confirmed the identity of the two products.

Sinulariol B (**9**), $C_{20}H_{34}O_2$, mp 61—63 °C, $[\alpha]_D$ −52° was also a cembrane diol and its ^{1}H-NMR spectrum showed a composite signal of three olefinic methyl protons at δ 1.57 and the signals of the olefinic protons on three trisubstituted double bonds at δ 5.11, 5.02 and 4.94 (each br t, J=7 Hz). This pattern was quite similar to those of **8a** and other simple cembrane-type hydrocarbons.[3] No signals due to isopropyl or isopropenyl groups were seen. Instead, the signals due to a deshielded tertiary methyl (δ 1.13, 3H, s) and a tertiary hydroxymethyl (3.44 and 3.55, each 1H, d, J=11.0 Hz) were present and indicated that C-15 and C-16 are oxygenated. The mass spectrum (MS) showed ions at *m/z* 306 (M^+), 288 (M^+ − Me) and 275 (M^+ − hydroxymethyl). Reduction of the monotosylate of **9** gave a tertiary alcohol, $[\alpha]_D$ −39°, which showed good agreement of ^{1}H-NMR and mass spectral properties with the natural product nephthenol (**11**), $[\alpha]_D$ −36°, which was isolated first from a soft coral, *Nephthea* sp.[10] Its absolute configuration was established.

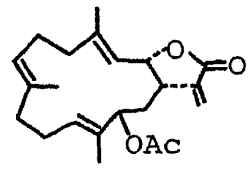
α-Methylene-γ-lactones in soft corals could reasonably be supposed to be derived from the simple hydrocarbon cembrene A, which is the cyclization product of geranylgeraniol. Compounds **8a** and **9** appear to be possible (not necessarily exclusive) intermediates, between cembrene A and α-methylene-γ-lactone derivatives of soft corals (Chart 3). The primary product would be the simple lactone **10**, which is known to occur in *S. mayi*.[4a]

Experimental

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Optical rotations were



1a: R=β-OAc, α-H
1b: R=β-OH, α-H
1c: R=O



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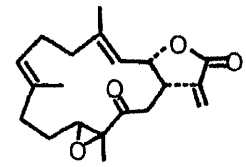

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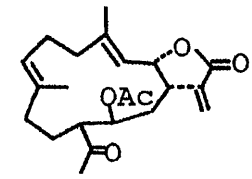

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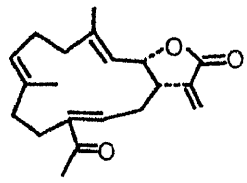

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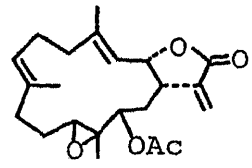

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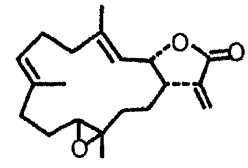

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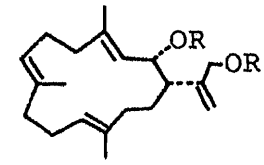
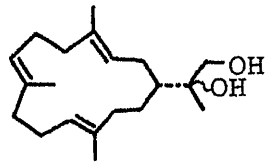


8a: R=H
8b: R=Ac



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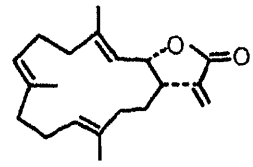

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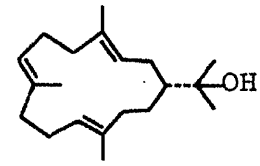


11

Chart 1

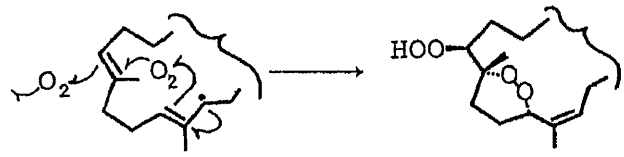
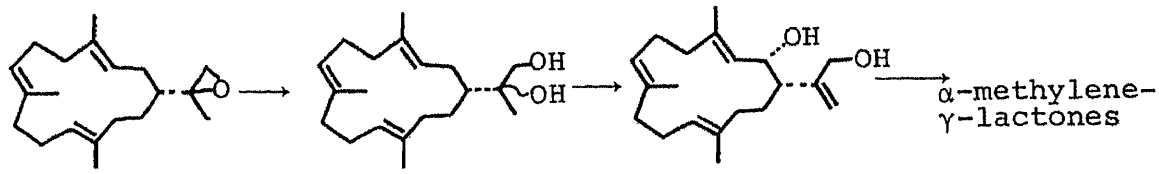


Chart 2



cembrene A oxide

Chart 3

determined on a JASCO DIP-4 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were determined, unless otherwise specified, on a JEOL-FX 200 spectrometer at 200 MHz and 50 MHz, in $CDCl_3$ solution with tetramethylsilane as an internal standard. Mass spectra were determined on JEOL JMS D-300 (EI-MS) and JEOL JMS 01SG-2 (FD-MS) spectrometers. IR spectra were taken on a JASCO A-102 spectrometer. Column chromatography was carried out by the flash chromatography method.

Fractionation of *S. mayi* Extract——Partially dried material (15.8 kg) of *S. mayi*, collected in June 1983 was extracted first with MeOH (10 l×3; 1054 g) and then with MeOH–$CHCl_3$ (10 l×5; 1026 g). The MeOH extract contained significant amounts of inorganic salts. The crude lipid was obtained by dissolving the MeOH extract in MeOH–$CHCl_3$ (1 : 1) and removing insoluble materials. A portion (178 g) of the crude lipid was charged on a column of silica gel (1.5 kg) and eluted successively with hexane (4 l), hexane–$CHCl_3$ (1 : 1, 25 l), $CHCl_3$ (11 l), MeOH–$CHCl_3$ (1 : 100, 25 l), and MeOH–$CHCl_3$ (3 : 7, 5.5 l). The fractions (1.5 l/fraction) were collected, monitored by thin-layer chromatography (TLC), and combined as follows: fr. 1 (a, 0.02 g), 2—3 (b, 0.12 g), 4—5 (c, 3.36 g), 6—7 (d, 6.25 g), 8—9 (e, 22.7 g), 10—12 (f, 17.35 g), 13—14 (g, 10.47 g), 15—17 (h, 11.99 g), 18—22 (i, 7.17 g), 23—24 (j, 4.22 g), 25—26 (k, 14.18 g), 27—30 (l, 7.38 g), 31—32 (m, 4.56 g), 33—35 (n, 9.83 g), 36—40 (o, 9.81 g), 41—44 (p, 4 g), 45 (q, 23.89 g), 46—47 (r, 25.75 g). Repeated flash chromatography of portions of fractions e, i, j, and k gave compounds **1**—**9** as follows. Chromatography of fraction e with EtOAc–hexane (1 : 3) gave **1** (650 mg) and **2** (220 mg). Chromatography of fraction i with EtOAc–hexane (2 : 3) gave **3** (165 mg) and **4** (3 g). Chromatography of fraction j with ether–$CHCl_3$ (1 : 50) gave **5** (30 mg), **6** (23 mg) and **7** (55 mg). Chromatography of fraction k with ether–$CHCl_3$ (1 : 9), MeOH–$CHCl_3$ (1 : 50) and EtOAc–hexane (2 : 3) gave **8a** (90 mg) and **9** (30 mg).

Compound 1——Colorless plates, mp 122—123 °C, $[\alpha]_D$ −18° (c=1.09, $CHCl_3$). ^{1}H-NMR δ: 1.09 (3H, s, 19-H), 1.67 (3H, br s, 18-H), 1.76 (3H, br s, 20-H), 1.83 (1H, ddd, J=13.7, 12.0, 2.4 Hz, 14-H), 2.11 (3H, s, OAc), 2.82 (1H, ddd, J=12.2, 6.5, 2.4 Hz, 1-H), 3.08 (1H, ddd, J=13.7, 11.3, 2.4 Hz, 14-H), 4.33 (1H, dd, J=12.1, 2.4 Hz, 11-H), 5.20 (1H, dd, J=7.3, 6.5 Hz, 2-H), 5.37 (1H, br d, J=11.3 Hz, 13-H), 5.50 (1H, d, J=7.3 Hz, 3-H), 5.63 (1H, d, J=1.5 Hz, 17-H), 5.78 (1H, d, J=9.8 Hz, 7-H), 6.25 (1H, d, J=1.5 Hz, 17-H). ^{13}C-NMR δ: 16.4 (q), 19.6 (q), 21.0 (q), 24.4 (q), 24.8 (t), 26.1 (t), 28.1 (t), 31.1 (t), 35.6 (t), 44.6 (d), 70.2 (d), 79.3 (d), 80.0 (s), 84.3 (d), 121.3 (t), 123.3 (d), 125.5 (d), 135.4 (s), 138.4 (s), 140.2 (s), 170.3 (s), 170.5 (s). UV λ_{max}^{EtOH} nm: 212. IR ν_{max}^{Nujol} cm^{-1}: 1765, 1755, 1730, 1660. MS m/z: 390 (M^+), 330, 307, 287, 269, 247. *Anal.* Calcd for $C_{22}H_{30}O_6$: C, 67.67; H, 7.74. Found: C, 67.36; H, 7.72.

Compound 2——Colorless oil, $[\alpha]_D$ +29° (c=0.96, $CHCl_3$). ^{1}H-NMR (δ): 1.56 (3H, s), 1.61 (3H, s), 1.76 (3H, s), 2.07 (3H, s, OAc), 3.11 (1H, ddd, J=11.7, 7.8, 2.9 Hz, 1-H), 4.70 (1H, br d, J=10.0 Hz), 4.91 (1H, d, J=10.7 Hz, 3-H), 5.05 (1H, d, J=11.2 Hz, 13-H), 5.25 (1H, br dd, J=10, 6 Hz), 5.50 (1H, d, J=3.3 Hz, 17-H), 5.54 (1H, dd, J=10.7, 7.8 Hz, 2-H), 6.26 (1H, d, J=3.9 Hz, 17-H). UV λ_{max}^{EtOH} nm: 209. IR ν_{max}^{Nujol} cm^{-1}: 1760, 1720, 1660. MS m/z: 358 (M^+), 298, 248.

Compound 3——Colorless oil, $[\alpha]_D$ −35° (c=1.03, $CHCl_3$). ^{1}H-NMR (δ): 1.62 (3H, s), 1.67 (3H, s), 1.43 (3H, s, 20-H), 2.63 (1H, dd, J=10.7, 19.5 Hz, 14-H), 2.73 (1H, dd, J=19.5, 3.9 Hz, 14-H), 3.00 (1H, dd, J=8.3, 3.5 Hz, 11-H), 3.63 (1H, m, 1-H), 4.82 (1H, br d, J=10.3 Hz, 3-H), 4.95 (1H, t, J=6.4 Hz, 7-H), 5.47 (1H, d, J=3.4 Hz, 17-H), 5.60 (1H, dd, J=10.2, 8.3 Hz, 2-H), 6.25 (1H, d, J=3.4 Hz, 17-H). UV λ_{max}^{EtOH} nm: 214. IR ν_{max}^{Nujol} cm^{-1}: 1760, 1705, 1660. MS m/z: 330 (M^+), 312, 259, 178, 165. High-resolution MS: Found 330.1826. Calcd for $C_{20}H_{26}O_4$ (M^+) 330.1832.

Compound 4——Colorless powder, mp 110—113 °C, $[\alpha]_D$ −76° (c=0.98, $CHCl_3$). ^{1}H-NMR (δ): 1.60 (3H, s), 1.87 (3H, s), 2.15 (3H, s), 2.20 (3H, s), 3.07 (1H, m, 1-H), 5.05 (1H, br d, J=10.0 Hz, 3-H), 5.10 (1H, m, 7-H), 5.19 (1H, dt, J=9.0, 3.2 Hz, 13-H), 5.40 (1H, dd, J=10.0, 8.1 Hz, 2-H), 5.53 (1H, d, J=2.7 Hz, 17-H), 6.24 (1H, J=3.2 Hz, 17-H). UV λ_{max}^{EtOH} nm: 213. IR ν_{max}^{Nujol} cm^{-1}: 1760, 1755, 1730, 1700, 1660. MS m/z: 374 (M^+), 332, 314, 296, 271. High-resolution MS: Found 374.2091. Calcd for $C_{22}H_{30}O_5$ (M^+) 374.2094.

Compound 5——Colorless oil, $[\alpha]_D$ +28° (c=1.3, $CHCl_3$). ^{1}H-NMR (δ): 1.63 (3H, s), 1.70 (3H, s), 2.15 (3H, s), 3.45 (1H, m, 1-H), 4.67 (1H, m, 7-H), 5.06 (1H, br d, J=8.8 Hz, 3-H), 5.45 (1H, t, J=8.7 Hz, 2-H), 5.62 (1H, d, J=2.2 Hz, 17-H), 6.38 (1H, d, J=2.2, 17-H), 6.37 (1H, t, J=5.2, 13-H). UV λ_{max}^{EtOH} nm: 214, 235. IR ν_{max}^{Nujol} cm^{-1}: 1750, 1665, 1660. MS m/z: 314 (M^+), 299, 296, 281, 271. High-resolution MS: Found 314.1882. Calcd for $C_{20}H_{26}O_3$ (M^+) 314.1882.

Compound 6——Colorless needles, mp 184—185 °C, $[\alpha]_D$ +56° (c=0.86, $CHCl_3$). ^{1}H-NMR (δ): 1.15 (3H, s, 20-H), 1.59 (3H, s), 1.87 (3H, s), 2.17 (3H, s), 2.78 (1H, dd, J=10, 3 Hz, 11-H), 2.95 (1H, m, 1-H), 4.93 (1H, br d, J=10.5 Hz, 3-H), 4.97 (1H, m, 7-H), 4.98 (1H, dd, J=11.5, 1.7 Hz, 13-H), 5.43 (1H, dd, J=10.3, 7.8 Hz, 2-H), 5.49 (1H, d, J=3.4 Hz, 17-H), 6.25 (1H, d, J=3.4 Hz, 17-H). UV λ_{max}^{EtOH} nm: 215. IR ν_{max}^{Nujol} cm^{-1}: 1760, 1730, 1670. MS m/z: 374 (M^+), 332, 315, 314, 299, 296. High-resolution MS: Found 374.2083. Calcd for $C_{22}H_{30}O_5$ (M^+) 374.2093.

Compound 7——Colorless needles, mp 126—127 °C, $[\alpha]_D$ +37° (c=0.73, $CHCl_3$). ^{1}H-NMR (δ): 1.18 (3H, s, 20-H), 1.61 (3H, s), 1.72 (3H, s), 2.75 (1H, dd, J=10, 2.7 Hz, 11-H), 3.06 (1H, m, 1-H), 5.01 (1H, d, J=6.4 Hz, 7-H), 5.07 (1H, d, J=9.8 Hz, 3-H), 5.30 (1H, dd, J=9.8, 8.3 Hz, 2-H), 5.54 (1H, d, J=2.6 Hz, 17-H), 6.27 (1H, d, J=2.9 Hz, 17-H). UV λ_{max}^{EtOH} nm: 209. IR ν_{max}^{Nujol} cm^{-1}: 1750, 1655. MS m/z: 316 (M^+).

Compound 8a——Colorless needles, mp 116—117.5 °C, $[\alpha]_D$ +101° (c=0.99, $CHCl_3$). ^{1}H-NMR (δ): 1.55 (3H, s), 1.58 (3H, s), 1.61 (3H, s), 4.03 (1H, d, J=12.7 Hz, 16-H), 4.17 (1H, d, J=12.7 Hz, 16-H), 4.17 (1H, d, J=12.7 Hz, 16-H), 4.50 (1H, dd, J=8.8, 1.5 Hz, 2-H), 4.95 (1H, br s, 17-H), 5.00 (1H, m), 5.03 (1H, m), 5.16 (1H, br s, 17 H), 5.43 (1H, d, J=8.8 Hz, 3-H). UV λ_{max}^{EtOH} nm: end absorption. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1660, 900. MS m/z: 304 (M^+), 286, 202. High-resolution MS: Found 304.2406. Calcd for $C_{20}H_{32}O_2$ (M^+) 304.2402.

Compound 9——Colorless needles, mp 61—63 °C, $[\alpha]_D$ −52° (c=1.12, $CHCl_3$). ^{1}H-NMR (δ): 1.13 (3H, s, 17-H), 1.57 (9H, s, 18-, 19-, 20-H), 3.44 (1H, d, J=11 Hz, 16-H), 3.55 (1H, d, J=11 Hz, 16-H), 4.94 (1H, t, J=7 Hz), 5.02 (1H, t, J=7 Hz), 5.11 (1H, t, J=7 Hz). UV λ_{max}^{EtOH} nm: end absorption. IR ν_{max}^{Nujol} cm^{-1}: 3150, 3250. MS m/z: 306 (M^+),

288, 275, 257, 189. High-resolution MS: Found 306.2555. Calcd for $C_{20}H_{34}O_2$ (M^+) 306.2558.

Hydrolysis and Oxidation of 1a——A solution of **1a** (40 mg) in 3 ml of *tert*-BuOH was mixed with 0.2 ml of 50% aq. KOH soln and stirred at 80 °C for 30 min. After cooling, the mixture was poured into 1 N HCl solution and extracted with Et_2O. After evaporation of the solvent, the residue was dissolved in 3 ml of 0.02 N H_2SO_4 in dioxane–H_2O (4:1) and stirred at 80 °C for 20 min. After the usual work-up, the mixture was purified by silica gel column chromatography to give 20 mg of the hydroxylactone (**1b**), mp 184.5—186.5 °C. ^{1}H-NMR (δ): 1.05 (3H, s, 19-H), 1.67 (3H, s, 18-H), 1.77 (3H, s, 20-H), 2.83 (1H, br dd, J=12.7, 6.5 Hz, 1-H), 4.34 (1H, dd, J=12.2, 2.0 Hz, 11-H), 4.35 (1H, d, J=11.2 Hz, 7-H), 5.21 (1H, t, J=6.5 Hz, 2-H), 5.32 (1H, br d, J=10.0 Hz, 13-H), 5.40 (1H, d, J=6.5 Hz, 3-H), 5.65 (1H, br s, 17-H), 6.23 (1H, br s, 17-H). UV λ_{max}^{EtOH} nm: 214. IR ν_{max}^{Nujol} cm^{-1}: 3500, 1765, 1655. MS *m/z*: 330 ($M^+ - H_2O$), 312, 43. Compound **1b** (10 mg) in Et_2O (3 ml) was stirred with 1 ml of Jones' reagent for 1 min and poured into H_2O. It was extracted with Et_2O and worked-up as usual. Purification of the product by silica gel column chromatography gave the ketone **1c** (8 mg), mp 117—120 °C. ^{1}H-NMR (δ): 1.26 (3H, s, 19-H), 1.67 (3H, s, 18-H), 1.76 (3H, s, 20-H), 4.40 (1H, dd, J=12.0, 2.0 Hz, 11-H), 5.14 (1H, dd, J=7.0, 5.4 Hz, 2-H), 5.29 (1H, m, 13-H), 5.40 (1H, d, J=7.0 Hz, 3-H). UV λ_{max}^{EtOH} nm: 214. IR ν_{max}^{Nujol} cm^{-1}: 1765, 1720, 1665. MS *m/z*: 346 (M^+), 328.

Acetylation of 8a——A mixture of **8a** (25 mg) in pyridine (0.5 ml) and acetic anhydride (0.15 ml) was left at room temperature overnight. The mixture was diluted with EtOH with cooling, and the solvent was evaporated off *in vacuo*. Silica gel column chromatography of the residue with 10% ethyl acetate in hexane gave 30 mg of diacetate **8b** as an oil, $[\alpha]_D$ +66° (c=1.03, $CHCl_3$). ^{1}H-NMR (δ): 1.57 (6H, s), 1.69 (3H, s), 2.00 (3H, s), 2.05 (3H, s), 4.43 (1H, d, J=13.2 Hz, 16-H), 4.62 (1H, d, J=13.2 Hz, 16-H), 5.03 (1H, br s, 17-H), 5.18 (1H, br s, 17-H), 5.29 (1H, br d, J=8.8 Hz, 3-H), 5.56 (1H, dd, J=8.8, 1.5 Hz, 2-H).

Conversion of 9 into Nephthenol (11)——A mixture of **9** (28.5 mg) and *p*-toluenesulfonyl chloride (50 mg) in pyridine was kept at room temperature for 1 h and the solvent was evaporated off *in vacuo* at room temperature. The product was extracted with Et_2O, then the Et_2O layer was washed with 5% HCl, H_2O and sat. NaCl solution, and the solvent was evaporated off. The residue was dissolved in tetrahydrofuran (1 ml) and stirred at room temperature with 15 mg of $LiAlH_4$. Excess $LiAlH_4$ was decomposed with moist Et_2O, the mixture was filtered, the Et_2O solution was washed with 5% HCl, H_2O and sat. NaCl solution, and the solvent was evaporated off. Chromatography of the residue (17.3 mg) over a column of silica gel with 7% ethyl acetate in hexane gave 12.3 mg of **11**, as an oil, $[\alpha]_D$ −39° (c=1.2, $CHCl_3$). ^{1}H-NMR (δ): 1.20 (6H, s), 1.56 (3H, s), 1.57 (6H, s), 4.95 (1H, br t, J=7.0 Hz), 5.01 (1H, br t, J=7.0 Hz), 5.11 (1H, br t, J=7.0 Hz). MS *m/z*: 290 (M^+), 272, 229, 217, 189, 175, 161.

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An Improved Synthesis of the New Angiotensin Converting Enzyme Inhibitor CV-5975 *via* a Chemoenzymatic Process

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A chemoenzymatic synthesis of the new angiotensin converting enzyme inhibitor CV-5975 (**1**) is described. The optically active key intermediate for the synthesis of **1**, ethyl (*R*)-6-(1-benzyloxycarbonyl-4-piperidyl)-2-hydroxyhexanoate ((*R*)-**4**), was prepared by kinetic resolution of the racemic α-hydroxyester ((*RS*)-**4**) with a lipase and also by asymmetric reduction of the α-oxoester (**3**) with baker's yeast. The enantiomeric excess (ee) of the α-hydroxyester ((*R*)-**4**) produced by these enzymatic procedures exceeded 60%. This optically active alcohol ((*R*)-**4**) was converted to its mesylate ((*R*)-**5**), which was then subjected to S_N2 reaction with the aminobenzothiazepine derivative (**2**) followed by deprotection to yield **1**.

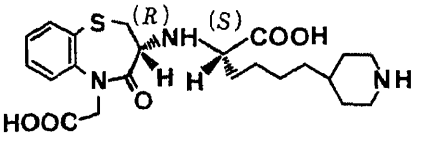
Keywords——ACE inhibitor; 1,5-benzothiazepine derivative; chemoenzymatic synthesis; enzymatic hydrolysis; baker's yeast reduction; CV-5975

Recently, we reported the discovery of the new angiotensin converting enzyme (ACE) inhibitor, (*R*)-3-[(*S*)-1-carboxy-5-(4-piperidyl)pentyl]amino-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepine-5-acetic acid (CV-5975; **1**), which shows potent and long-lasting *in vivo* inhibitory activity.[1a–c] This compound includes two asymmetric carbons, with (*R*)-configuration of the 3-position and (*S*)-configuration in the side chain, both of which have been confirmed to be essential for the high biological activity.[1c] Introduction of (*R*)-chirality into the 3-position of the benzothiazepine skeleton, leading to the optically pure intermediate **2** (ee, >99%),[2] was achieved by using a natural amino acid, L-cysteine, as the starting material as described in our earlier report.[1a] However, little asymmetric induction was observed in the reductive alkylation of **2** with the α-oxoester **3** in the presence of sodium cyanoborohydride ($NaBH_3CN$), producing a diastereomeric mixture of (*R*),(*S*)-**6** and (*R*),(*R*)-**6** (*ca.* 1 : 1) in 19% yield.[1c]

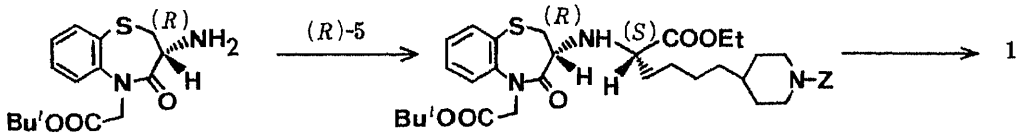
A more efficient and practical route to **1** was needed in order to make the compound more available for further biological studies. In our previous work,[1c] we found that a substitution reaction of the α-methanesulfonyloxyester (*RS*)-**5** with **2** proceeded smoothly to yield a diastereomeric mixture of diesters ((*R*),(*S*)-**6** and (*R*),(*R*)-**6**) in good yield. This suggested that if the (*R*)-α-methanesulfonyloxyester (*R*)-**5** could be obtained, it would react with **2** in an S_N2 process without racemization, thus giving the desired diester (*R*),(*S*)-**6** exclusively.[3,4]

This report describes an improved synthesis of CV-5975 (**1**) which utilizes chemoenzymatic methods to prepare the key chiral fragment, ethyl (*R*)-6-(1-benzyloxycarbonyl-4-piperidyl)-2-hydroxyhexanoate ((*R*)-**4**).

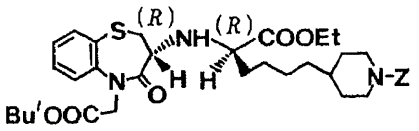
Initially, we attempted to resolve the racemic α-hydroxyacid (*RS*)-**7** by salt formation with a variety of chiral amines[5] available commercially. Although quinine and cinchonidine formed crystalline salts of the acid, complete resolution could not be attained by recrystallization because there were only small differences in the relative solubilities. This



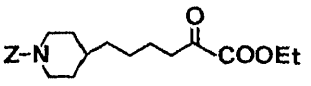
1 : CV-5975



2 (*R*), (*S*)-6



(*R*), (*R*)-6



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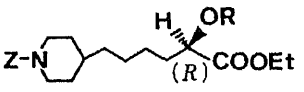
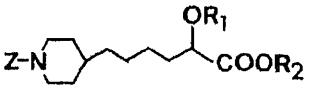
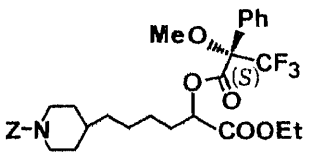


(*R*)-4 : R=H
(*R*)-5 : R=Ms



(*RS*)-4 : R_1=H, R_2=Et
(*RS*)-5 : R_1=Ms, R_2=Et
(*RS*)-7 : R_1=H, R_2=H



8

Bu^t=*tert*-Bu
Z=$COCH_2Ph$

Chart 1

unsuccessful approach prompted us to examine kinetic resolution[6] of the racemic α-hydroxyester (*RS*)-**4** utilizing hydrolytic enzymes to produce the chiral α-hydroxyester (*R*)-**4**. First, we screened a variety of hydrolytic enzymes[7] for activity towards the substrate (*RS*)-**4** and found that lipase M-AP10 (from *Mucor*)[8] and lipase PN (from *Phycomyces nitens*)[9] could hydrolyze the ester moiety of (*RS*)-**4** without any side reactions. Next, the relation between the conversion (%) and the optical purity (% ee) of the remaining substrate fraction was investigated with lipase M-AP10 and lipase PN. The hydrolysis was carried out with vigorous mixing of the substrate (*RS*)-**4** and the enzyme in 0.1 M phosphate buffer (pH 6.8) at 30 °C.[10] At suitable intervals, the conversion (%) was monitored by analyzing an aliquot of the reaction mixture by high-performance liquid chromatography (HPLC). The enantiomeric excess (% ee) of the unhydrolyzed ester in the sample was also determined by HPLC after formation of the corresponding (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid

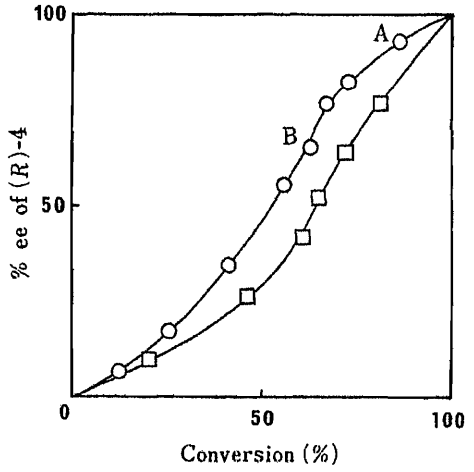


Fig. 1. The Relation between Conversion (%) and % ee of the Remaining α-Hydroxyester ((*R*)-**4**)

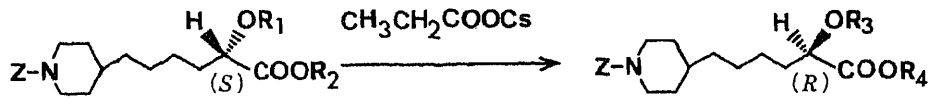
—○—, lipase PN; —□—, lipase M-AP10.

(MTPA) ester **8**.[11] As shown in Fig. 1,[12] lipase PN showed a slightly higher stereoselectivity than lipase M-AP10. The absolute configuration of the remaining unreacted α-hydroxyester was assigned as *R* as follows: highly optically active ester (93% ee) was obtained by stopping the hydrolysis when the conversion reached 86% (lipase PN, point A in Fig. 1). This ester was converted to the mesylate **5** which was allowed to react with **2** (90 °C, 20 h) to yield the diester **6**, which proved to be identical with (*R*),(*S*)-**6** on HPLC. Furthermore, this product showed high diastereomeric excess (de, 93%), which corresponded to 93% ee of the α-hydroxyester **4**. These findings indicated that complete Walden inversion had occurred at the α-position of the mesylate in this S_N2 process and therefore, the configuration of the α-hydroxyester should be *R*.

To use the resolved ester in the practical synthesis of **1**, we scaled up the procedure to a run with 40 g of the substrate (*RS*)-**4**. The reaction was stopped at 63% conversion (point B, in Fig. 1) to obtain (*R*)-**4** in a suitable chemical yield (14.6 g, 37%). The use of this (*R*)-enriched α-hydroxyester (64% ee) allowed us to omit chromatographic separation of the diastereomers **6** in the course of synthesis of CV-5975 (**1**). Treatment of this (*R*)-**4** with mesyl chloride (pyridine, 0 °C) afforded the mesylate (*R*)-**5**, which was allowed to react with **2** to yield the diester in 85% yield with 80:20 ((*R*),(*S*)-**6**: (*R*),(*R*)-**6**) diastereomeric ratio. Deprotection of this diester (HBr–AcOH; aqueous NaOH) gave CV-5975 (**1**), which was readily purified by recrystallization from water.

The above enzyme system hydrolyzed the (*S*)-ester to produce the (*S*)-acid ((*S*)-**7**), leaving the exploitable (*R*)-enriched ester ((*S*)-**4**) behind. Thus, methods are also needed to utilize the (*S*)-acid ((*S*)-**7**) for the synthesis of **1**. This was achieved by Swern oxidation[13] of its ester ((*S*)-**4**) into the α-oxoester **3**, which could be reduced to obtain the starting substrate (*RS*)-**4**.[1c] Also, hydroxy group inversion of the (*S*)-ester ((*S*)-**4**, 74% ee) could be accomplished *via* Kellogg's method[14] ((*S*)-**7**→(*S*)-**4**→(*S*)-**5**→(*R*)-**9**→(*R*)-**7**→(*R*)-**4**) and the Mitsunobu reaction[15] ((*S*)-**7**→(*S*)-**4**→(*R*)-**10**) as shown in Chart 2. These inversions proceeded without perceptible racemization as shown by comparison of ee on HPLC or the specific rotation of the materials (*S*-configuration) with that of the products (*R*-configuration).

Our next approach to preparing the (*R*)-hydroxyester (*R*)-**4** was asymmetric reduction of the α-oxoester **3**. One of the most promising methods for the preparation of an optically active alcohol seems to be baker's yeast reduction of a carbonyl compound.[16] This method is very versatile for organic synthesis because it is simple and economical as well as highly enantioselective. The stereochemistry of the alcohol produced by baker's yeast reduction can be predicted by the so-called Prelog rule[17] in the case of β-oxocarboxylic acid derivatives.[16]



(*S*)-7 : $R_1=R_2=H$
(*S*)-4 : $R_1=H$, $R_2=Et$
(*S*)-5 : $R_1=Ms$, $R_2=Et$

(*R*)-9 : $R_3=COCH_2CH_3$, $R_4=Et$
(*R*)-7 : $R_3=R_4=H$
(*R*)-4 : $R_3=H$, $R_4=Et$

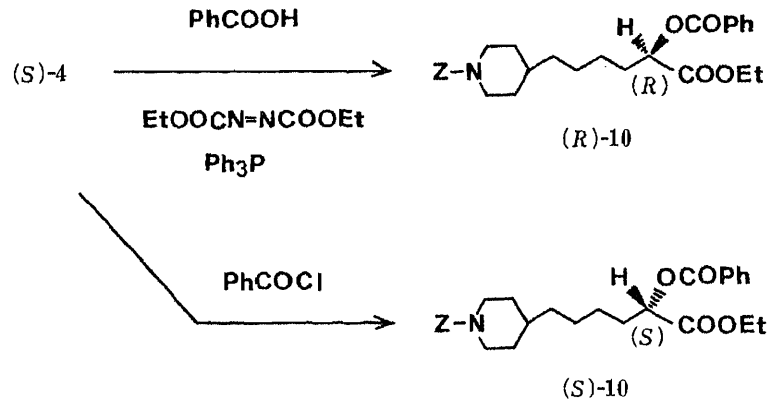


Chart 2

TABLE I. Baker's Yeast Reduction of α-Oxoesters (3, 11a—c)

3, 11a—c (*R*)-4, 12a—c

Entry	α-Oxoester R	(g)	Reaction time (h)	(*R*)-α-Hydroxyester yield (%)[a]	((*R*)-4, 12a—c) % ee
1	Me (**11a**)	(1)	22	52	37
2	Et (**3**)	(1)	46	34	54
3	Pr (**11b**)	(1)	42	43	32
4	Bu (**11c**)	(1)	66.5	31	30
5	Et (**3**)	(40)	48	46	61

a) Isolated yield.

However, few studies have been done with α-oxocarboxylic acid derivatives. Iriuchijima and Ogawa reported[18] that the yeast reduction of methyl 4-amino-*N*-benzyloxycarbonyl-2-oxobutanoate having a structure similar to that of our substrate **3** gave (*S*)-α-hydroxyester in 49% ee, which suggests that it is an anti-Prelog product. Hence, it is difficult to predict the stereochemical course in the baker's yeast reduction of an α-oxoester such as **3**. Fortunately, **3** gave the desired (*R*)-hydroxyester (*R*)-**4** as shown in Table I. The run with 1 g of the substrate **3** gave (*R*)-**4** in 34% chemical yield with 54% ee (entry 2).

With baker's yeast reduction of β-oxoesters, the optical purity of the product varies with the size of the ester moiety.[19] To improve the ee in the α-oxoester reduction, we prepared a series of esters, *i.e.* methyl, propyl and butyl ester (**11a—c**), and exposed them to baker's yeast

(entries 1, 3 and 4). No improvement of the optical purity was observed. However, in scaling up the run to 40 g of **3**, a slight improvement in optical purity was observed when the substrate was added to the fermenting suspension over a period of 10 h. (*R*)-**4** was obtained in 46% chemical yield with 61% ee (entry 5). This ester was also used in the synthesis of CV-5975 (**1**) as shown in Chart 1.

In summary, we established a procedure using chemoenzymatic processes, lipase hydrolysis and baker's yeast reduction, to prepare the new angiotensin converting enzyme inhibitor CV-5975 (**1**). This new route can be used to prepare **1** in sufficient quantities for biological investigations.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus (a hot stage type) and are uncorrected. The infrared (IR) spectra were recorded with a Hitachi 260-10 spectrophotometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Varian EM-360, EM-390 and XL-100A instruments in the indicated solvents. Chemical shifts are reported as δ-values relative to tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were obtained on a JEOL JMS-01SC mass spectrometer. The $[\alpha]_D$ values were determined in the indicated solvents on a JASCO DIP 181 4-4822. HPLC analyses were performed on a Shimadzu LC-5A instrument equipped with an SPD-2A detector operating at 254 nm. Peak areas were calculated by using a Shimadzu C-R3A Chromatopac.

Reactions were run at room temperature unless otherwise noted, and followed by thin-layer chromatography (TLC) on Merck Silica gel F_{254} and RP-8F_{254S} plates. Standard work-up procedures were as follows. The reaction mixture was partitioned between the indicated solvent and water. The organic extract was washed in the indicated order with water, $NaHCO_3$ solution (aq. $NaHCO_3$), NaOH solution (aq. NaOH) and hydrochloric acid (aq. HCl), then dried over $MgSO_4$, filtered and evaporated *in vacuo*. Chromatographic separation was done on Merck Silica gel 60 using the indicated eluents.

(*RS*)-6-(1-Benzyloxycarbonyl-4-piperidyl)-2-hydroxyhexanoic Acid ((*RS*)-7)——A solution of NaOH (18 g) in water (200 ml) was added to a solution of (*RS*)-**4** (114 g) in EtOH (100 ml) over a period of 15 min. The mixture was stirred for 20 min, then water (500 ml) was added. The mixture was acidified with conc. HCl and worked up (hexane–AcOEt (1 : 1); water). The oily residue was crystallized from Et_2O to give (*RS*)-**7** (67 g, 64%) as colorless crystals, mp 90—94 °C. IR ν_{max}^{Nujol} cm^{-1}: 3500 (OH), 1730, 1650 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 1.0—1.9 (12H, m), 2.5—3.1 (3H, m), 3.9—4.4 (3H, m), 5.1 (2H, s, CH_2Ph), 7.0 (1H, br, OH), 7.3 (5H, s, phenyl protons). *Anal.* Calcd for $C_{19}H_{27}NO_5$: C, 65.31; H, 7.79; N, 4.01. Found: C, 65.18; H, 7.92; N, 4.04.

Enzymatic Hydrolysis of Ethyl (*RS*)-6-(1-Benzyloxycarbonyl-4-piperidyl)-2-hydroxyhexanoate (Fig. 1)——A mixture of (*RS*)-**4** (5 g), lipase M-AP10 (600 mg) and 0.1 M phosphate buffer (260 ml) was vigorously stirred at 30 °C. After being stirred for 1.5 h, an aliquot (20 ml) of the reaction mixture was taken out, acidified with 1 N aq. HCl and extracted with AcOEt. To determine the conversion (%) of hydrolysis, a small portion of the extract was concentrated *in vacuo* and the residue was analyzed by HPLC under the following conditions: column (Zorbax ODS, 0.46 × 25 cm), mobile phase (water : MeOH = 1 : 5); flow rate (0.3 ml/min) and retention times (20 min for the α-hydroxyester **4** and 14 min for the α-hydroxyacid **7**). The main portion of the extract was washed with aq. $NaHCO_3$ and water, dried over $MgSO_4$ and concentrated *in vacuo* to give the unreacted α-hydroxyester **4**. This alcohol was converted in the usual manner[11] to the corresponding (*S*)-MTPA ester **8**, which was analyzed by HPLC under the following conditions: column (Zorbax ODS, 0.46 × 25 cm); mobile phase (water : MeOH = 1 : 8); flow rate (0.3 ml/min) and retention times (29 min for (*R*),(*S*)-**8** and 32 min for (*S*),(*S*)-**8**). Monitoring of the reaction as described above was repeated after the mixture had been stirred for 3, 4, 5, 6 and 8 h.

Hydrolysis of (*RS*)-**4** (5 g) with lipase PN (25 mg) was carried out similarly to the case of lipase M-AP10, and determination of the relation between conversion (%) and % ee by HPLC was done in the same manner as above.

Stereochemical Assignment of the Remaining Ester in Enzymatic Hydrolysis——The α-hydroxyester **4** (100 mg) with 93% ee, which was obtained by stopping the hydrolysis at point A (lipase PN, 86% conversion) in Fig. 1, was allowed to react with MsCl as described previously[1c] to give the mesylate **5** (115 mg, 95%). A mixture of the mesylate **5** (30 mg) and **2** (51 mg) was heated at 90 °C for 20 h. After cooling, the reaction mixture was worked up (AcOEt; 5% phosphoric acid, water) to give the diester **6** (38 mg, 86%). This diester **6** was analyzed by HPLC under the following conditions: column (Zorbax ODS); mobile phase (water: MeOH = 1 : 8); flow rate (0.3 ml/min); retention times (26 min for (*R*),(*R*)-**6** and 21 min for (*R*),(*S*)-**6**). HPLC analysis showed that the main peak was identical with (*R*),(*S*)-**6** and the ratio of the peak areas of (*R*),(*S*)-**6** and (*R*),(*R*)-**6** was 96.5 : 3.5.

CV-5975 (1)——A mixture of (*RS*)-**4** (40 g), lipase PN (40 mg) and 0.1 M phosphate buffer (1.2 l) was stirred vigorously at 30 °C for 3.5 h. The mixture was acidified with conc. HCl and worked up (AcOEt; water, aq. $NaHCO_3$, water) to give (*R*)-**4** (14.6 g, 37%) in 64% ee. This alcohol (7 g) was converted to the mesylate **5** (8.2 g, 97%) in the

usual manner. $[\alpha]_D^{23.5}$ +15.0° (c=0.52, MeOH). A mixture of this mesylate **5** (8.0 g) and **2** (13.5 g) was heated at 90 °C for 29 h. After cooling, the reaction mixture was worked up (AcOEt; 5% phosphoric acid; water). The oily residue was dissolved in a mixture of oxalic acid (4 g) and Et_2O (50 ml), and the solution was diluted with petroleum ether (200 ml). After cooling, the supernatant layer was removed by decantation, and AcOEt (300 ml) and aq. $NaHCO_3$ (150 ml) were added to the precipitate. The resulting mixture was shaken thoroughly and worked up (AcOEt; water) to give the diester **6** (10 g, 60% de) as an oil. Then 30% HBr–AcOH (30 ml) was added to a solution of this diester **6** in AcOH (15 ml). The mixture was allowed to stand for 2 h and diluted with Et_2O (250 ml). After standing, the supernatant layer was removed by decantation. The residue was dissolved in 1 N aq. NaOH (130 ml). After standing for 1 h, the solution was weakly acidified with AcOH and purified by MCI gel (Mitsubishi Chemical Industries, Ltd., CHP20p, 150—300 μ) chromatography (water : MeOH = 1 : 1). The eluate was concentrated (*ca.* 5 ml) *in vacuo* and allowed to stand overnight. The deposited crystals were collected by filtration to give **1** (2.05 g, 25% based on (*R*)-**4**), which was identical with CV-5975 prepared previously.[1c]

Swern Oxidation of the α-Hydroxyester——The $NaHCO_3$-washing in the enzymatic hydrolysis of (*RS*)-**4** (40 g) with lipase PN was acidified with conc. HCl and worked up (AcOEt; water) to give the (*S*)-α-hydroxyacid (*S*)-**7** (23.5 g, 63%). A mixture of this (*S*)-**7** (22 g), EtOH (50 ml), *p*-toluenesulfonic acid (1.0 g) and toluene (300 ml) was heated at 80—90 °C overnight. After cooling, the mixture was worked up (AcOEt; aq. $NaHCO_3$, water) to give (*S*)-**4** (24 g, 99%) in 32% ee.

A solution of dimethyl sulfoxide (DMSO) (4.45 g) in CH_2Cl_2 (20 ml) was added to a solution of oxalyl chloride (2.3 ml) in CH_2Cl_2 (40 ml) at −65 °C (acetone–dry ice bath) over a period of 10 min, and the mixture was stirred at −60 °C for 10 min. A solution of (*S*)-**4** (5 g, 32% ee) in CH_2Cl_2 (50 ml) was added over a period of 10 min, and the resulting mixture was stirred at −60 °C for 20 min. Diethylisopropylamine (10.3 g) was added over a period of 10 min, and then the acetone–dry ice bath was removed. When the temperature reached −30 °C, 1 N aq. HCl (80 ml) was added to the mixture. After being stirred for 30 min, the mixture was worked up. The residue was purified by silica gel column chromatography (hexane : AcOEt = 4 : 1—2 : 1) to give **3** (2.6 g, 52%)[20] as an oil, which was identical with **3** prepared previously.[1c]

Hydroxy Group Inversion of (*S*)-4 by Kellogg's Method——A mixture of (*S*)-**4** (10 g, 32% ee), lipase PN (20 mg) and 0.1 M phosphate buffer (300 ml) was stirred for 3 h. The mixture was acidified with conc. HCl and extracted with AcOEt (300 ml). The AcOEt layer was washed with aq. $NaHCO_3$ (150 ml). The aqueous layer was acidified with conc. HCl and worked up (AcOEt; water) to give (*S*)-**7** (3.8 g, 41%). This (*S*)-**7** was converted to the ethyl ester in the same manner as described above to give (*S*)-**4** (3.7 g) in 74% ee. This ester (*S*)-**4** (1.0 g) was converted to the mesylate (*S*)-**5** (1.2 g, 99%). $[\alpha]_D^{24.5}$ −16.6° (c=0.5, MeOH).

A solution of propionic acid (0.32 g) in MeOH (4 ml) was added to a solution of Cs_2CO_3 (0.47 g) in MeOH (12 ml). After being stirred for 30 min, the mixture was concentrated *in vacuo*. Toluene (20 ml) was added to the residue, and the mixture was evaporated *in vacuo*. After repeated addition of toluene followed by evaporation (several times), the resulting white powder (CH_3CH_2COOCs) was mixed with (*S*)-**5** (1.2 g) obtained above and *N*,*N*-dimethylformamide (DMF) (30 ml). The mixture was heated at 90 °C for 1 h and worked up (AcOEt; 0.1 N aq. HCl, aq. $NaHCO_3$, water). The oily residue was subjected to silica gel column chromatography (hexane : AcOEt = 3 : 1) to give (*R*)-**9** (1.07 g, 94%) as an oil. IR ν_{max}^{neat} cm^{-1}: 1745, 1700 (C=O). $[\alpha]_D^{24.5}$ +12.2° (c=0.74, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.15 (3H, t, J=7 Hz, CH_3), 1.25 (3H, t, J=7 Hz, CH_3), 0.7—2.2 (13H, m), 2.4 (2H, q, J=7 Hz, $COCH_2$), 2.7—3.0 (2H, m, NCH×2), 3.9—4.4 (4H, m, OCH_2, NCH×2), 4.95 (1H, t, J=6 Hz, OCHCOO), 5.15 (2H, s, CH_2Ph), 7.3 (5H, s, phenyl protons).

Then 1 N aq. NaOH (10 ml) was added to a solution of (*R*)-**9** (1.05 g) in EtOH (10 ml) over a period of 10 min. The resulting mixture was stirred for 30 min, acidified with conc. HCl and worked up (AcOEt; water) to give (*R*)-**7**. This (*R*)-**7** was esterified to (*R*)-**4** (0.82 g, 92%) in a usual manner using EtOH and TsOH. The ee value of this (*R*)-**4** was 70% as determined by HPLC analysis.

Ethyl (*S*)-2-Benzoyloxy-6-(1-benzyloxycarbonyl-4-piperidyl)hexanoate ((*S*)-10)——Benzoyl chloride (0.09 ml) was added to a solution of (*S*)-**4** (0.2 g, 74% ee) in pyridine (2 ml) at 0 °C, and the mixture was stirred at 0 °C for 30 min. After addition of water (1 ml), the mixture was stirred for 1 h and worked up (AcOEt; 1 N aq. HCl, aq. $NaHCO_3$, water) to give (*S*)-**10** (0.25 g, 98%) as an oil. $[\alpha]_D^{24}$ −6.0° (c=0.65, MeOH). IR ν_{max}^{neat} cm^{-1}: 1750, 1720, 1695 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7 Hz, CH_3), 0.7—2.3 (13H, m), 2.7 (2H, t, J=15 Hz, NCH×2), 3.9—4.4 (4H, m, NCH×2, OCH_2), 5.1 (2H, s, CH_2Ph), 5.2 (1H, t, J=7 Hz, OCHCOO), 7.2—8.3 (10H, m, phenyl protons).

Mitsunobu Reaction of (*S*)-4——A mixture of (*S*)-**4** (0.7 g, 74% ee), benzoic acid (0.45 g), triphenyl phosphine (0.97 g), diethyl azodicarboxylate (0.65 g) and tetrahydrofuran (THF) (15 ml) was refluxed overnight. The mixture was concentrated *in vacuo* and the residue was subjected to silica gel column chromatography (hexane : AcOEt = 4 : 1—2 : 1) to give (*R*)-**10** (0.38 g, 52%) as an oil. $[\alpha]_D^{25}$ +5.8° (c=0.4, MeOH).

α-Oxoesters (11a—c)——A mixture of (*RS*)-**7** (7 g), MeOH (20 ml), *p*-toluenesulfonic acid (0.3 g) and toluene (100 ml) was heated at 80—90 °C overnight. After cooling, the reaction mixture was worked up (AcOEt; aq. $NaHCO_3$, water) to give methyl 6-(1-benzyloxycarbonyl-4-piperidyl)-2-hydroxyhexanoate (*RS*)-**12a** (6.6 g, 98%) as an oil. IR ν_{max}^{neat} cm^{-1}: 3430 (OH), 1735, 1690 (C=O). ^{1}H-NMR ($CDCl_3$) δ: 0.7—2.0 (13H, m), 2.5—2.95 (2H, m,

NCH × 2), 2.95—3.15 (1H, br d, OH), 3.75 (3H, s, CH_3), 3.9—4.4 (3H, m, NCH × 2, CHCOO), 5.1 (2H, s, CH_2Ph), 7.3 (5H, s, phenyl protons).

The propyl (**12b**) and butyl (**12c**) esters were prepared similarly using PrOH and BuOH, respectively.

(*RS*)-**12b**: Yield 80%. IR ν_{max}^{neat} cm^{-1}: 3450 (OH), 1730, 1690 (C=O).

(*RS*)-**12c**: Yield 73%. IR ν_{max}^{neat} cm^{-1}: 3450 (OH), 1730, 1695 (C=O).

The α-hydroxyesters (**12a—c**) prepared above were converted to the corresponding α-oxoesters (**11a—c**) by Swern oxidation in a manner similar to that described for the preparation of **3**.

11a: Yield 40%. IR ν_{max}^{neat} cm^{-1}: 1730, 1690 (C=O). MS *m/z*: 361 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.7—2.0 (11H, m), 2.5—3.0 (4H, m), 3.85 (3H, s, CH_3), 3.95—4.4 (2H, m, NCH × 2), 5.1 (2H, s, CH_2Ph), 7.4 (5H, s, phenyl protons).

11b: Yield 45%. IR ν_{max}^{neat} cm^{-1}: 1725, 1695 (C=O). MS *m/z*: 389 (M^+).

11c: Yield 75%. IR ν_{max}^{neat} cm^{-1}: 1725, 1695 (C=O). MS *m/z*: 403 (M^+).

Baker's Yeast Reduction of the α-Oxoesters (Table I)——Entries 1—4: A mixture of baker's yeast (Oriental dry yeast, 10 g), sucrose (20 g) and water (100 ml) was stirred at 30 °C for 10 min. A solution of an α-oxoester (1.0 g) in EtOH (3 ml) was added to the mixture and the resulting mixture was stirred at 30 °C. After 16 h, sucrose (5 g) was added to the mixture and the stirring was continued for a further 30 h. After addition of AcOEt (100 ml), the mixture was filtered through a Celite pad. The AcOEt layer was separated, and the aqueous layer was extracted with AcOEt (100 ml). The extracts were combined and washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to silica gel column chromatography (hexane : AcOEt = 2 : 1) to give the corresponding α-hydroxyester as an oil, the ee value of which was determined by the same method as described in the case of lipase hydrolysis.

Entry 5: A mixture of baker's yeast (200 g), sucrose (100 g) and water (2.5 l) was stirred for 10 min. A solution of **3** (40 g) in EtOH–water (3 : 1, 200 ml) and a solution of sucrose (200 g) in water (1 l) were added simultaneously over a period of 10 h. After the addition was complete, the stirring was continued for 38 h, and the mixture was filtered through a Celite pad. The filtrate was extracted with AcOEt–petroleum ether (2 : 1, 750 ml × 2). Insoluble materials were washed with EtOH (1 l × 3), and the EtOH solution was concentrated *in vacuo*. The residue and the extract were combined, and the resulting organic solution was washed with water, 0.1 N aq. NaOH (300 ml) and water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified on silica gel (hexane : AcOEt = 2 : 1) to give (*R*)-**4** (18.4 g, 46%) with 61% ee.

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References and Notes

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2) The optical purity of **2** was determined by HPLC analysis of the corresponding (*S*)-MTPA amide on Zorbax SIL (hexane : THF : MeOH : H_2O = 40 : 1 : 0.1 : 0.01).
3) Initially we tested the possibility of S_N2-inversion reaction in this system using **2** and ethyl 2-mesyloxylactate. The reaction of (*S*)-mesyloxylactate with **2** (100—110 °C, 3 h) gave a single product which was identical with the (*R*),(*R*)-diester[1a] on TLC (hexane : AcOEt = 2 : 1), while the reaction of the racemic mesyloxylactate provided a diastereomeric mixture (*ca.* 1 : 1).
4) Successful S_N2-inversion reactions of chiral α-sulfonates have been reported: *a*) V. F. Effenberger, U. Burkard and J. Willfahrt, *Angew. Chem.*, **95**, 50 (1983); *b*) H. Urbach and R. Henning, *Tetrahedron Lett.*, **25**, 1143 (1984); *c*) R. M. Scott, Japan. Patent 59-59120 (1977) [*Chem. Abstr.*, **87**, 67990*n* (1977)].
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7) Lipase A and pancreatin are inactive in this hydrolysis.
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20) The chemical yield of the product **3** was slightly reduced by dimerization during the purification by silica gel chromatography. Afterwards, it was found that when silica gel was treated with AcOH before use (a mixture of silica gel (700 g), AcOH (20 ml) and hexane–CH_2Cl_2 (1:1, 500 ml) was evaporated thoroughly *in vacuo*), dimerization of **3** was suppressed.

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Synthesis of Head Activator (HA)-Related Peptides and Development of HA-Radioimmunoassay

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Synthesis of peptides related to hydra head activator (HA; pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH) was performed by the solution method. The analogs synthesized were Tyr^{11}-HA, des-Phe^{11}-HA, Arg^{1}-HA and Phe^{5}-HA. Antisera were prepared by immunizing three rabbits with the synthetic HA. Two anti-HA-antisera with sufficient titer were obtained. An HA-radioimmunoassay system, developed by using an antiserum (ASH-04) and ^{125}I–Tyr^{11}-HA as a tracer, was found to be sensitive and specific to HA.

Keywords——head activator; synthesis; antibody preparation; ^{125}I–Tyr^{11}-head activator; radioimmunoassay; specificity; droplet counter current chromatography; HPLC

The hydra head activator (HA) is an undecapeptide isolated from the fresh water hydra and the sea anemone, and sequenced as pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH[1] (I). The peptide is a growth hormone-like factor in hydra stimulating head specific growth or bud formation, together with other morphogenous factors.[2] A peptide with complete sequence homology occurs not only in the coelenterates but also in mammals—human, bovine and rat hypothalami, and intestine.[3] Except for the induction of amylase release from rat pancreas *in vitro* but not *in vivo*, and a weak stimulatory effect on smooth muscle,[4] no biological activity or physiological function of HA has been reported in mammals.

In the present paper we describe: a) the synthesis of four HA-related peptides (Fig. 1), and b) the development of an HA-radioimmunoassay (RIA).

Synthesis of HA-related peptides was carried out by a conventional solution method. The protected peptides prepared as units for successive azide fragment condensation to build up the peptide backbones were as follow; Z–pGlu–Pro–Pro–Gly–NHNHBoc (VI), Z–Arg(NO_2)–Pro–Pro–Gly–NHNHBoc,[5] Boc–Gly–Ser–N_2H_3,[6] Boc–Phe–Ser–N_2H_3,[7] Boc–Lys(Z)–Val–Ile–Leu–Tyr–OH (VII), Boc–Lys(Z)–Val–Ile–Leu–OH (VIII) and Boc–Lys(Z)–Val–Ile–Leu–Phe–OH (IX). In Fig. 2, the synthetic route is shown for an analog, Tyr^{11}-HA (II), which

	1 9 10 11
HA (I)	pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH
Tyr^{11}-HA (II)	pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Tyr–OH
des-Phe^{11}-HA (III)	pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–OH
Arg^{1}-HA (IV)	H–Arg–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH
Phe^{5}-HA (V)	pGlu–Pro–Pro–Gly–Phe–Ser–Lys–Val–Ile–Leu–Phe–OH
bradykinin (BK)	H–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg–OH

Fig. 1. Head Activator (HA) and Related Peptides

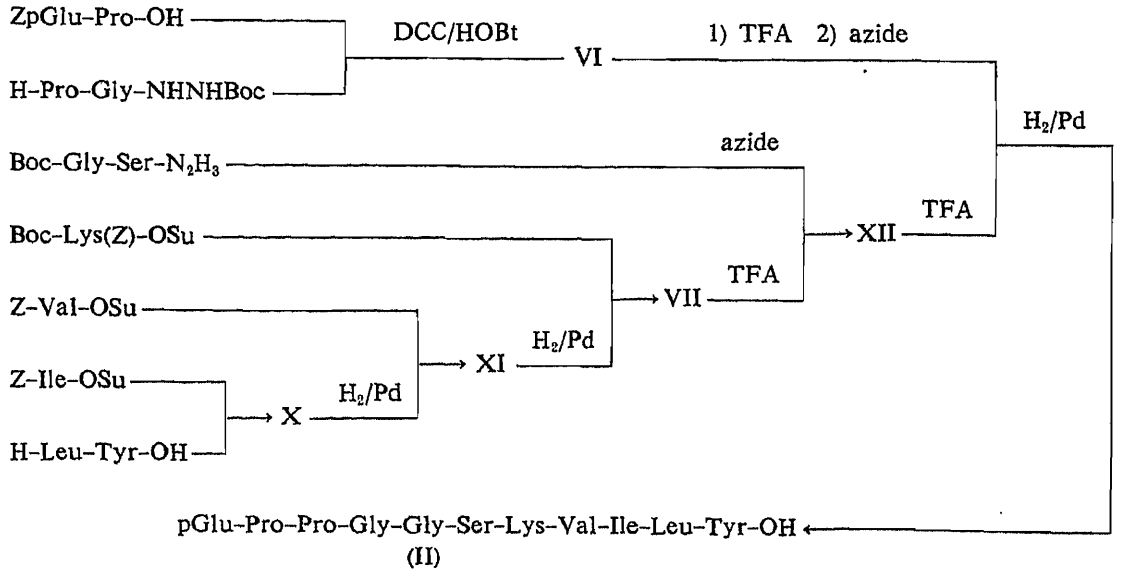

Fig. 2. Synthesis of Tyr[11]-HA (II)

was designed as a labelled antigen preparation with minimum structural modification (replacing C-terminal Phe by Tyr). The C-terminal protected pentapeptide (VII) was prepared in a stepwise fashion by the active ester method. The starting material, H–Leu–Tyr–OH[8] was coupled with Z–Ile–OSu to yield Z–Ile–Leu–Tyr–OH (X). Debenzyloxycarbonylation of X by catalytic hydrogenation followed by coupling with Z–Val–OSu gave Z–Val–Ile–Leu–Tyr–OH (XI), which was deprotected by the same procedure. Acylation of the resulting tetrapeptide with the active ester of Boc–Lys(Z)–OH gave VII. Removal of the N^{α}–Boc group of VII was carried out by treatment with trifluoroacetic acid (TFA) at 15 °C for 45 min to suppress N^{ε}–Z cleavage of Lys.[9] The resulting pentapeptide H–Lys(Z)–Val–Ile–Leu–Tyr–OH·TFA (VII') was condensed with Boc–Gly–Ser–N_2H_3[6] by the azide method[10] and the protected heptapeptide Boc–Gly–Ser–Lys(Z)–Val–Ile–Leu–Tyr–OH (XII) obtained was further subjected to de-*tert*-butyloxycarbonylation under the same conditions as described above.

The N-terminal tetrapeptide (VI) was prepared by the reaction between Z–pGlu–Pro–OH[11] and H–Pro–Gly–NHNHBoc[5] with the dicyclohexylcarbodiimide (DCC)–*N*-hydroxybenzotriazole (HOBt) procedure.[12] The final coupling of Z–pGlu–Pro–Pro–Gly–N_3 derived from VI with H–Gly–Ser–Lys(Z)–Val–Ile–Leu–Tyr–OH (XII') yielded the protected undecapeptide Z–pGlu–Pro–Pro–Gly–Gly–Ser–Lys(Z)–Val–Ile–Leu–Tyr–OH, which was hydrogenated to give crude II. Purification of peptide II was performed by droplet counter current chromatography (DCCC),[13] with a solvent system of *n*-butanol–acetic acid–water (4 : 1 : 5). The desired peptide emerged after the solvent front of the lower phase used as the mobile phase. A highly purified product was obtained after gel filtration on Sephadex G-25 using 2 M acetic acid as the solvent. The homogeneity of this peptide was proved by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), elemental analysis and amino acid analysis of the acid hydrolysate.

The other three HA analogs, des-Phe[11]-HA (III), Arg[1]-HA (IV) and Phe[5]-HA (V), were synthesized in the same manner as described above, starting from VIII or IX as the C-terminal fragment. HA itself was also prepared and extensively purified by DCCC employing two different solvent systems. The homogeneity of the synthetic peptides was proved by the same means as used for II. The synthetic HA prepared in this study had the same biological activity as the native peptide. It also cross-reacted with the HA-RIA system developed by Bodenmüller *et al.*[14] Cross-reactivity and homogeneity data are summarized in Table I.

TABLE I. Characterization of Synthetic Peptides

Peptide	Rf^1	Rf^2	Retention time[a] (min)	Cross-reactivity (%)
I	0.17	0.59	12.35	100
II	0.13	0.56	8.71	100
III	0.11	0.48	7.43	1
IV	0.02	0.53	9.53	0.03
V	0.25	0.64	16.06	0.3

a) Conditions of analytical HPLC: column, μ-Bondapak C_{18} (4×300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 25% to 45% CH_3CN (15 min) in 20 mM phosphate buffer (pH 3.0).

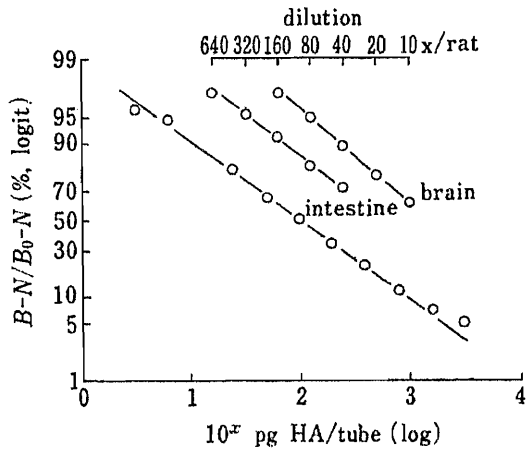

Fig. 3. Standard Dose-Response Curve and Dilution Plots of 2 M AcOH Extract from Whole Intestine and Whole Brain of Rat

Antiserum, ASH-04 (final 20000×); tracer, ^{125}I-Tyr^{11}-HA; F/B separation, double antibody method.

To obtain a specific antiserum, we used HA-ascaris protein conjugates,[15] prepared by using glutaraldehyde. Immunization of three rabbits with the conjugate gave antibodies with sufficient titer. ^{125}I-Tyr^{11}-HA was prepared by the Iodogen method.[16] Purified labelled antigen was separated from the unlabelled antigen and other compounds by reversed-phase HPLC.

The specificity of these antisera was examined, and the antiserum ASH-04 was found to be more specific in binding to the undecapeptide HA than the others. In the HA-RIA using ASH-04 at a final dilution of 20000 fold, the cross-reactivities of the three HA analogs and bradykinin (BK) were examined. The dose-response curves of III, IV and V, which resemble BK, revealed that the minor change of the HA molecule resulted in a drastic decrease of the binding activity with the antisera (Table I). The Gly^5 residue seemed to be important for binding with the antibody ASH-04, while BK did not cross-react at all. These results clearly indicate that the assay system is specific for the undecapeptide. In HA-RIA by the double antibody method, less than 10 pg of HA per tube was detectable; 2 N acetic acid extracts of rat whole intestine and whole brain displaced the tracer in a manner parallel to that of synthetic HA (Fig. 3). The content of immunoreactive HA, however, was found to be extremely low. The results suggest that the RIA system established here could be useful for further immunological studies on the head activator.

Experimental

Melting points are uncorrected. Optical rotations were measured in a Nippon Bunkoh DIP-4 polarimeter. Amino acid analyses were performed with a Hitachi KLA-5 amino acid analyzer. HPLC was performed on a system

composed of two model 510 pumps, a model U6K injector, a model 680 gradient controller, a model 481 LC spectrophotometer and a data module, model 730 (Waters). Radioactivity was counted with an Auto Well Gamma System, ARC-360 (Aloka). Acid hydrolysis of samples for amino acid analysis was conducted with twice-distilled 5.8 N HCl at 110 °C for 24 h and for 48 h in evacuated sealed tubes, and phenol was added thereto when the peptide contained tyrosine. Evaporation of solvents was carried out *in vacuo* below 45 °C in a rotary evaporator. The solvent systems used for TLC on silica gel (Merck) were *n*-BuOH–AcOH–H_2O (4 : 1 : 5, the upper phase, Rf^1) and *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24, Rf^2).

Z–Ile–Leu–Tyr–OH (X)——Z–Ile–OSu (2.21 g, 6.1 mmol) was allowed to react at room temperature with H–Leu–Tyr–OH[8] (1.38 g, 4.7 mmol) in dimethylformamide (DMF) (15 ml)–H_2O (6 ml) containing triethylamine (TEA) (1.32 ml, 9.4 mmol) for 16 h. After evaporation of the solvents, the residue in AcOEt was washed successively with 1 N HCl and saturated NaCl solution. The organic phase was dried over Na_2SO_4 and evaporated. The residue was triturated with ether–petr. ether and the solid was reprecipitated from the same solvents. Yield 2.47 g (96.9%). mp 123—125 °C. Rf^1 0.85, Rf^2 0.82. $[\alpha]_D^{21}$ −0.07° (c = 1.0, DMF). *Anal.* Calcd for $C_{29}H_{39}N_3O_7$: C, 64.31; H, 7.26; N, 7.76. Found: C, 63.92; H, 7.30; N, 7.57.

Z–Val–Ile–Leu–Tyr–OH (XI)——X (2.33 g, 4.3 mmol) was hydrogenated in *n*-BuOH (40 ml)–MeOH (40 ml)–10% AcOH (80 ml) over Pd-black for 20 h. The catalyst was removed by filtration and the filtrate was concentrated to a small volume. The residue was precipitated from EtOH. Yield 1.75 g (87.0%). Rf^1 0.77, Rf^2 0.80. Z–Val–OSu (1.42 g, 4.1 mmol) was coupled with H–Ile–Leu–Tyr–OH (1.59 g, 3.4 mmol) obtained above in H_2O (6 ml)–dimethylsulfoxide (DMSO) (2 ml)–DMF (20 ml) containing TEA (0.81 ml, 5.8 mmol) at room temperature for 20 h. The solvents were evaporated off and the residue was triturated with 10% AcOH. The solid was reprecipitated from MeOH–ether. Yield 1.73 g (79.4%). mp 232—233 °C. Rf^1 0.87, Rf^2 0.75. $[\alpha]_D^{21}$ −2.6° (c = 1.0, DMF). *Anal.* Calcd for $C_{34}H_{48}N_4O_8$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.37; H, 7.57; N, 8.46.

Boc–Lys(Z)–Val–Ile–Leu–Tyr–OH (VII)——XI (1.60 g, 2.5 mmol) was hydrogenated in *n*-BuOH (15 ml)–MeOH (60 ml)–50% AcOH (30 ml) in the same manner as described for XI. Yield 1.26 g (88.7%). Rf^1 0.75, Rf^2 0.80. Boc–Lys(Z)–OSu [prepared from Boc–Lys(Z)–OH (0.989 g, 2.6 mmol), *N*-hydroxysuccinimide (HOSu) (0.314 g, 2.73 mmol), and DCC (0.563 g, 2.73 mmol) in DMF (1 ml)–THF (10 ml) as usual] was coupled with the tetrapeptide obtained above in H_2O (8 ml)–DMSO (15 ml)–DMF (40 ml) at room temperature for 20 h. The mixture was treated in the same manner as described for XI and reprecipitated from AcOEt–ether. Yield 0.58 g (39.1%). mp 275—277 °C (dec.). Rf^1 0.85, Rf^2 0.81. $[\alpha]_D^{21}$ −11.1° (c = 0.7, DMF). *Anal.* Calcd for $C_{45}H_{68}N_6O_{11} \cdot 3/2H_2O$: C, 60.32; H, 7.99; N, 9.38. Found: C, 60.41; H, 7.85; N, 9.55.

Boc–Gly–Ser–Lys(Z)–Val–Ile–Leu–Tyr–OH (XII)——VII (0.56 g, 0.64 mmol) was dissolved in cold TFA (5 ml) containing anisole (0.5 ml) and the solution kept at 15 °C for 45 min. Excess of TFA was evaporated off and anhydrous Et_2O was added to the residue. The precipitate VII′ was washed with Et_2O and dried over KOH/P_2O_5. Rf^1 0.78, Rf^2 0.76. Isoamyl nitrite (0.18 ml, 1.34 mmol) was added to a solution of Boc–Gly–Ser–N_2H_3 (0.35 g, 1.28 mmol) in DMF (8 ml) containing 6 N HCl in dioxane (0.640 ml, 3.84 mmol) at −15 °C. This mixture was stirred at −10 °C for 2 min and neutralized with an ice-cold solution of TEA (0.538 ml, 3.84 mmol) in DMF (3 ml), and then was combined with an ice-cold solution of VII′ in DMSO (7 ml)–DMF (4 ml) containing TEA (0.179 ml, 1.28 mmol). The reaction mixture was kept for 1 h at −5 °C and for 24 h at 4 °C and then concentrated. The product was precipitated in the same manner as described for XI and reprecipitated with MeOH–AcOEt–ether. Yield 0.48 g (73.8%). mp 284—285 °C (dec.). Rf^1 0.92, Rf^2 0.89. $[\alpha]_D^{13}$ −9.1° (c = 1, DMF). *Anal.* Calcd for $C_{50}H_{76}N_8O_{14} \cdot 1/2H_2O$: C, 58.75; H, 7.59; N, 10.96. Found: C, 58.69; H, 7.41; N, 10.95.

Z–pGlu–Pro–Pro–Gly–NHNH–Boc (VI)——DCC (1.14 g, 5.5 mmol) was added to an ice-cold solution of Z–pGlu–Pro–OH[11] (1.80 g, 5 mmol), H–Pro–Gly–NHNHBoc[5] (5 mmol) [prepared from Z–Pro–Gly–NHNHBoc[5] (2.10 g) by catalytic hydrogenation] and HOBt (0.74 g, 5.5 mmol) in DMF (5 ml)–tetrahydrofuran (THF) (25 ml). The mixture was stirred for 40 h at 4 °C and the resulting solid was filtered off. The filtrate was concentrated to a small volume and the residue was distributed between five portions of *n*-BuOH and ten portions of H_2O. The combined upper phase was concentrated to give a residue, which was passed through a column of Sephadex LH-20 (3.2 × 90 cm) with MeOH–1% AcOH (9 : 1) as the eluent. The fractions (8 g each, No. 35—37) were concentrated. The product was triturated with ether. Yield 2.09 g (66.6%). mp 151—155 °C (dec.). Rf^1 0.57, Rf^2 0.79. $[\alpha]_D^{15}$ −99.0° (c = 1.0, DMF). *Anal.* Calcd for $C_{30}H_{40}N_6O_9$: C, 57.32; H, 6.41; N, 13.37. Found: C, 57.20; H, 6.44; N, 13.19. Amino acid ratios in an acid hydrolysate (24 h): Glu 1.00, Pro 2.00, Gly 1.00.

pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Tyr–OH (II)——XII (101 mg, 0.1 mmol) was partially deprotected with TFA–anisole at 15 °C in the same manner as described for XII to give H–Gly–Ser–Lys(Z)–Val–Ile–Leu–Tyr–OH · TFA (XII′). Rf^1 0.55, Rf^2 0.71. VI (107 mg, 0.17 mmol) was treated with TFA (5 ml) at 20 °C for 45 min in the same manner as described for XII to give Z–pGlu–Pro–Pro–Gly–N_2H_3 · TFA (VI′). Rf^1 0.41, Rf^2 0.66. The product VI′ was converted to the azide in DMF (3 ml) by using 6 N HCl in dioxane (0.283 ml, 1.7 mmol) and isoamyl nitrite (23 μl, 0.17 mmol) in the same manner as described for XII. The azide was coupled with XII′ (0.1 mmol) in DMSO (5 ml)–DMF (5 ml) at 4 °C for 15 h. The product was precipitated from 20% AcOH (80 ml), washed with MeOH (1 ml)–AcOEt (10 ml) (yield 85 mg; Rf^1 0.43, Rf^2 0.79), and hydrogenated over Pd in *n*-BuOH (5 ml)–MeOH (25 ml)–50% AcOH (5 ml). The product was gel-filtered on a Sephadex G-25 column with 2 M AcOH as the eluent and

lyophilized. Yield 65 mg. The crude material (20 mg) was dissolved in a mixture (2 ml) of the upper and lower phases of *n*-BuOH–AcOEt–H_2O (4 : 1 : 5) and subjected to DCCC which employed 200 tubes (0.2 × 30 cm each) filled with the upper phase. The lower phase (350 ml) was pumped through the tubes from top to bottom at a flow rate of 3 ml/h, followed by the upper phase (300 ml). The fractions (3 g each, No. 66—120) were gel-filtered and lyophilized. Yield 17 mg (48.4%). $[\alpha]_D^{21}$ −115.6° (c=1, 3 M AcOH). *Anal.* Calcd for $C_{54}H_{84}N_{12}O_{10}\cdot 2AcOH\cdot 2H_2O$: C, 53.69; H, 7.46; N, 12.95. Found: C, 53.44; H, 6.90; N, 12.99. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.94 (1.01), Ser 0.80 (0.85), Glu 0.97 (1.05), Pro 2.34 (2.04), Gly 2.00 (2.06), Val 0.58 (0.78), Ile 0.54 (0.77), Leu 1.01 (0.97), Tyr 0.95 (1.02), NH_3 trace (0.30).

Z–Val–Ile–Leu–OH (XIII)——Z–Val–OSu (2.68 g, 7.7 mmol) was allowed to react with H–Ile–Leu–OH·HCl[17] (1.97 g, 7.0 mmol) in DMF (20 ml) containing TEA (2.66 ml, 19 mmol) at room temperature for 18 h. The product was worked up in the same manner as described for X and precipitated from AcOEt–ether. The solid was reprecipitated from the same solvents. Yield 3.13 g, (93.7%). mp 214—216 °C (dec.). Rf^1 0.93, Rf^2 0.86. $[\alpha]_D^{25}$ −10.0° (c=1.0, DMF). *Anal.* Calcd for $C_{25}H_{39}N_3O_6$: C, 62.87; H, 8.23; N, 8.80. Found: C, 63.00; H, 8.25; N, 8.73.

Boc–Lys(Z)–Val–Ile–Leu–OH (VIII)——XIII (995 mg, 2 mmol) was hydrogenated in MeOH (100 ml)–5% AcOH (50 ml) in the same manner as described for XI. The product (Rf^1 0.61, Rf^2 0.73) was reacted with Boc–Lys(Z)–OSu (2.6 mmol) in DMF (20 ml)–H_2O (5 ml) containing TEA (0.433 ml, 3.1 mmol) at room temperature for 20 h. The product was worked up in the same manner as described for XI and reprecipitated from AcOEt–ether. Yield 1.10 g (78.0%). mp 164—167 °C. Rf^1 0.91, Rf^2 0.86. $[\alpha]_D^{25}$ −22.4° (c=1.0, DMF). *Anal.* Calcd for $C_{35}H_{59}N_5O_9$: C, 61.26; H, 8.42; N, 9.92. Found: C, 60.97; H, 8.53; N, 10.02. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.03 (0.90), Val 0.80 (1.02), Ile 0.74 (0.97), Leu 0.97 (1.10), NH_3 0.33 (0.26).

Boc–Gly–Ser–Lys(Z)–Val–Ile–Leu–OH (XIV)——VIII (1.19 g, 1.4 mmol) was partially deblocked with ice-chilled TFA (5 ml)–anisole (0.5 ml) in the same manner as described for XII to give H–Lys(Z)–Val–Ile–Leu–OH·TFA (VIII'). Rf^1 0.75, Rf^2 0.74. The product was allowed to react with the azide [prepared from Boc–Gly–Ser–N_2H_3[6] (0.58 g, 2.1 mmol), 6 N HCl in dioxane (1.12 ml, 8.0 mmol) and isoamyl nitrite (0.28 ml, 2.1 mmol)] in DMSO (30 ml)–DMF (20 ml) at 4 °C for 18 h. The desired product was isolated in the same manner as described for XI and reprecipitated from MeOH (40 ml)–AcOEt (80 ml). Yield 1.04 g (87.5%). mp 226—227 °C (dec.). Rf^1 0.85, Rf^2 0.85. $[\alpha]_D^{25}$ −14.5° (c=1.0, DMF). *Anal.* Calcd for $C_{41}H_{67}N_7O_{12}\cdot H_2O$: C, 56.73; H, 8.01; N, 11.30. Found: C, 57.00; H, 8.14; N, 11.07. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.93 (1.08), Ser 0.95 (0.85), Gly 1.06 (1.07), Val 0.69 (1.01), Ile 0.80 (0.92), Leu 1.06 (1.00), NH_3 0.49 (0.45).

pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–OH (III)——XIV (128 mg, 0.15 mmol) was partially deblocked in the same manner as described for XII to give H–Gly–Ser–Lys(Z)–Val–Ile–Leu–OH·TFA (XIV'). Rf^1 0.53, Rf^2 0.74. The azide [prepared from VI' (0.22 mmol), 6 N HCl in dioxane (178 μl, 1.10 mmol) and isoamyl nitrite (30 μl, 0.22 mmol) in DMF (4 ml)] was allowed to react with XIV' in DMSO (4 ml)–DMF (6 ml) containing TEA (55 μl, 0.39 mmol) at 4 °C for 20 h, then the mixture was concentrated. The resulting residue was triturated with 5% AcOH, reprecipitated from MeOH–AcOEt (115 mg, Rf^1 0.47, Rf^2 0.77 as main spot), and hydrogenated in *n*-BuOH (15 ml)–MeOH (15 ml)–10% AcOH (15 ml). The product in 2 M AcOH was applied to a column (3.0 × 103 cm) of Sephadex G-25 (fine). The fractions (8 g each, No. 51—59) were lyophilized. Yield 41 mg (45.0%). Rf^1 0.11, Rf^2 0.48. $[\alpha]_D^{25}$ −117.6° (c=1.0, 3 M AcOH). *Anal.* Calcd for $C_{45}H_{75}N_{11}O_{13}\cdot AcOH$: C, 54.37; H, 7.67; N, 14.84. Found: C, 54.35; H, 7.40; N, 14.59. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.01 (1.08), Ser 0.91 (0.82), Glu 1.05 (1.05), Pro 2.02 (2.02), Gly 2.06 (2.07), Val 0.79 (0.98), Ile 0.74 (0.90), Leu 0.95 (0.95), NH_3 0.54 (0.65).

Z–Ile–Leu–Phe–OH (XV)——Z–Ile–OSu (6.12 g, 16.9 mmol) was allowed to react at room temperature for 20 h with H–Leu–Phe–OH[18] (3.62 g, 13 mmol) in DMF (25 ml)–H_2O (10 ml) containing TEA (3.09 ml, 22.1 mmol). The mixture was treated in the same manner as described for X. The residue was triturated with ether–petr. ether. The solid was reprecipitated from AcOEt–ether. Yield 5.34 g (76.8%). mp 156—158 °C. Rf^1 0.88, Rf^2 0.78. $[\alpha]_D^{15}$ −5.7° (c=1.0, DMF). *Anal.* Calcd for $C_{29}H_{39}N_3O_6\cdot 1/2H_2O$: C, 65.15; H, 7.54; N, 7.86. Found: C, 65.15; H, 7.50; N, 7.73.

Z–Val–Ile–Leu–Phe–OH (XVI)——XV (3.48 g, 6.5 mmol) was hydrogenated in MeOH (40 ml)–50% AcOH (20 ml) in the same manner as described for XI. The product (Rf^1 0.66, Rf^2 0.73) was precipitated from EtOH and allowed to react with Z–Val–OSu (2.72 g, 7.8 mmol) at room temperature for 20 h in H_2O (5 ml)–DMSO (5 ml)–DMF (40 ml) containing TEA (1.54 ml, 11 mmol). XVI was isolated in the same manner as described for XI and reprecipitated from MeOH–ether. Yield 3.17 g (76.9%). mp 227—228 °C. Rf^1 0.86, Rf^2 0.78. $[\alpha]_D^{15}$ −9.5° (c=1.0, DMF). *Anal.* Calcd for $C_{34}H_{48}N_4O_7\cdot 1/2H_2O$: C, 64.43; H, 7.79; N, 8.84. Found: C, 64.77; H, 7.89; N, 8.94.

Boc–Lys(Z)–Val–Ile–Leu–Phe–OH (IX)——XVI (1.90 g, 3 mmol) was hydrogenated in MeOH (40 ml)–*n*-BuOH (20 ml)–50% AcOH (20 ml) in the same manner as described for XI. The product (Rf^1 0.66, Rf^2 0.78) in DMSO (20 ml)–DMF (20 ml) containing TEA (0.56 ml, 6 mmol) was coupled with Boc–Lys(Z)–OSu (6 mmol) at room temperature. After 18 h, the mixture was treated in the same manner as described for XI and the product was reprecipitated from MeOH–ether. Yield 2.03 g (79.4%). mp 295—297 °C (dec.). Rf^1 0.89, Rf^2 0.74. $[\alpha]_D^{15}$ −15.7° (c=1.0, DMF). *Anal.* Calcd for $C_{45}H_{68}N_6O_{10}$: C, 63.36; H, 8.03; N, 9.85. Found: C, 63.00; H, 8.06; N, 9.88.

Boc–Gly–Ser–Lys(Z)–Val–Ile–Leu–Phe–OH (XVII)——IX (1.11 g, 1.3 mmol) was partially deblocked with ice-chilled TFA (10 ml)–anisole (1.3 ml) in the same manner as described for XII to give H–Lys(Z)–Val–Ile–Leu–Phe–OH·TFA (IX'). Rf^1 0.75, Rf^2 0.73. IX' in DMSO (30 ml)–DMF (15 ml) was allowed to react with azide [prepared

from Boc-Gly-Ser-N_2H_3 (0.72 g, 2.6 mmol), 6 N HCl in dioxane (1.73 ml, 10.4 mmol) and isoamyl nitrite (0.35 ml, 2.6 mmol)] at 4 °C for 20 h. The mixture was treated in the same manner as described for XI and the product was washed with MeOH. Yield 1.00 g (76.8%). mp 275—279 °C (dec.). Rf^1 0.86, Rf^2 0.80. $[\alpha]_D^{15}$ −13.9° (c=1.0, DMF). *Anal.* Calcd for $C_{50}H_{76}N_8O_{13}$: C, 60.22; H, 7.68; N, 11.24. Found: C, 59.77; H, 7.70; N, 11.35. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.99 (0.82), Gly 1.06 (0.98). Val 0.61 (0.73), Ile 0.63 (0.78), Leu 1.03 (0.97), Phe 0.98 (0.91), Lys 0.93 (1.15), NH_3 0.25 (0.43).

H-Arg-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH (IV)——Z-Arg(NO_2)-Pro-Pro-Gly-NHNHBoc[5] (144 mg, 0.2 mmol) was treated with TFA (3 ml) in the usual manner. The product was converted to the corresponding azide with 6 N HCl in dioxane (0.167 ml, 1 mmol) and isoamyl nitrite (0.027 ml, 0.2 mmol) in DMF (4 ml) and coupled at 4 °C for 22 h with H-Gly-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH·TFA (XVII'), which was derived from XVII (100 mg, 0.1 mmol) in the same manner as described for XII. The mixture was concentrated. The residue was precipitated from 20% AcOH and reprecipitated from MeOH-AcOEt. Yield 106 mg, Rf^1 0.55, Rf^2 0.73. The solid was hydrogenated over Pd and purified by DCCC using a solvent system of *n*-BuOH-AcOH-H_2O (4:1:5) in the same manner as described for II. The lower phase fractions (2 g each, No. 11—31) were gel-filtered on a Sephadex G-25 column. Yield 57 mg (49.0%). $[\alpha]_D^{13}$ −99.3° (c=1, 1 M AcOH). *Anal.* Calcd for $C_{55}H_{99}N_{15}O_{13}\cdot 3AcOH\cdot 3H_2O$: C, 51.80; H, 7.82; N, 14.96. Found: C, 51.80; H, 7.41; N, 15.16. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.69 (0.75), Pro 1.85 (1.92), Gly 1.86 (2.19), Val 0.67 (0.96), Ile 0.64 (0.95), Leu 0.86 (1.06), Phe 0.89 (1.04), Lys 1.12 (0.98), Arg 1.14 (1.13), NH_3 0.34 (0.58).

Boc-Phe-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH (XVIII)——The azide [prepared from Boc-Phe-Ser-N_2H_3[7] (257 mg, 0.70 mmol), 6 N HCl in dioxane (0.55 ml, 3.5 mmol), and isoamyl nitrite (0.094 ml, 0.70 mmol) in DMF (30 ml) in the usual manner] was coupled with IX' [prepared from IX (0.299 g, 0.35 mmol) in the same manner as described for XII] at 4 °C for 20 h. The product was isolated in the same manner as described for XI and reprecipitated from DMF-MeOH. Yield 0.32 g (83.0%). Rf^1 0.85, Rf^2 0.78. mp 292—294 °C (dec.). $[\alpha]_D^{15}$ −11.2° (c=1, DMF). *Anal.* Calcd for $C_{57}H_{82}N_8O_{13}$: C, 62.96; H, 7.60; N, 10.31. Found: C, 62.63; H, 7.70; N, 10.41. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.92 (0.80), Val 0.52 (0.74), Ile 0.57 (0.75), Leu 0.97 (1.02), Phe 1.94 (1.88), Lys 1.09 (1.08), NH_3 0.20 (0.17).

pGlu-Pro-Pro-Gly-Phe-Ser-Lys-Val-Ile-Leu-Phe-OH (V)——XVIII (218 mg, 0.2 mmol) was treated with TFA (5 ml)-anisole (0.5 ml) in the usual manner to give H-Phe-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH·TFA (XVIII'). Rf^1 0.72, Rf^2 0.70. The azide [prepared from VI' (0.34 mmol) as described for II] was coupled with XVIII' in DMSO (20 ml)-DMF (20 ml) at 4 °C for 48 h. The product was isolated in the same manner as described for XI, washed with MeOH-AcOEt [yield 190 mg; Rf^1 0.72, Rf^2 0.67 (main spot)], hydrogenated over Pd in *n*-BuOH-MeOH-50% AcOH (20 ml : 20 ml : 10 ml) and gel-filtered on Sephadex G-25 to give crude V (41 mg), which was subjected to DCCC using *n*-BuOH-AcOH-H_2O (4:1:5) in the same manner as described for II. After elution with the lower phase (300 ml), fractions (4 g each, No. 112—121) of the upper phase eluate were gel-filtered on Sephadex G-25. Yield 30 mg (15.0%). $[\alpha]_D^{15}$ −113.0° (c=0.5, 3 M AcOH). *Anal.* Calcd for $C_{61}H_{90}N_{12}O_{14}\cdot AcOH\cdot 2H_2O$: C, 57.70; H, 7.53; N, 12.82. Found: C, 57.47; H, 7.26; N, 13.03. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 1.17 (1.08), Glu 0.89 (0.97), Pro 1.95 (1.99), Gly 0.93 (1.06), Val 0.56 (0.87), Ile 0.59 (0.85), Leu 0.98 (1.03), Phe 2.02 (1.95), Lys 0.85 (1.01), NH_3 0.15 (0.27).

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH (I)——The azide [prepared from VI' (1.0 mmol)] was coupled with XVII' (0.5 mmol) in DMSO (15 ml)-DMF (10 ml) at 4 °C for 20 h. The product was isolated in the same manner as described for XI, and washed with MeOH-AcOEt. Yield 632 mg (90.7%). Rf^1 0.48, Rf^2 0.78 (main spot). The solid (500 mg) was hydrogenated in *n*-BuOH-MeOH-10% AcOH (1:1:1) (60 ml) over Pd and lyophilized from 1 M AcOH. Yield 401 mg. Crude I (100 mg) was subjected to DCCC using a solvent system of *n*-BuOH-AcOH-H_2O (4:1:5) in the same manner as described for II. The fractions (4 g each, No. 92—121) were concentrated to a small volume and passed through a column (3.2×90 cm) of Sephadex G-25 (fine) using 2 M AcOH as the eluent. The fractions (8 g each, No. 45—51) were lyophilized. Yield 68 mg. The product was then subjected to DCCC again using a solvent system of *n*-BuOH-pyridine-0.1% AcOH (5:3:11). The lower phase (300 ml) was used as the mobile phase. The fractions (4 g each, No. 65—85) were concentrated, lyophilized, and passed through a Sephadex G-25 column. The product was lyophilized from 2 M AcOH. Yield 56 mg (36.0%). $[\alpha]_D^{15}$ −127.5° (c=1, 3 M AcOH). *Anal.* Calcd for $C_{54}H_8N_{12}O_{14}\cdot AcOH\cdot 3H_2O$: C, 54.27; H, 7.64; N, 13.56. Found: C, 54.52; H, 7.30; N, 13.95. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.93 (0.81), Glu 1.05 (1.05), Pro 1.98 (1.94), Gly 2.06 (1.95), Val 0.61 (0.82), Ile 0.58 (0.75), Leu 1.02 (0.99), Phe 0.99 (0.98), Lys 1.02 (1.05), NH_3 0.14 (0.38).

Immunochemical Experiments

Preparation of Conjugate——A 2% aqueous solution of glutaraldehyde (1 ml) was added dropwise to an ice-cold solution of HA (7.5 mg) and crude protein extract of *Ascaris suilla* (15 mg) in H_2O (7.5 ml) during 30 min. The mixture was stirred overnight at 4 °C and taken into dialysis tubing (Spectrapor, MW cut-off 1000). The dialysis was continued at 4 °C for 20 h with two changes of H_2O (1 l each) and the dialysate was lyophilized. Yield 13 mg.

Immunization——The conjugate (3 mg) was dissolved in saline (1.5 ml) and the solution was emulsified with complete Freund's adjuvant (1.5 ml, Calbiochem-Behring) in a Sorval Omni-Mixer at 50000 rpm. The emulsion was injected subcutaneously at multiple sites on three mixed-bred male rabbits. The rabbits were boosted three times at

biweekly intervals and monthly thereafter using a half amount of the conjugate. Blood was taken at 10 d after the injection. All rabbits produced antibodies after the 5th injection.

Labelled Antigen Preparation——A solution of II (11 μg, 9.2 nmol) in H_2O (22 μl) was added to a solution of ^{125}INa (250 μCi, 0.115 nmol) in 0.25 M phosphate buffer (50 μl, pH 7.40) in a glass tube, which had been filmed with 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycouril (0.99 μg, 2.3 nmol, Iodogen, Pierce) at the bottom.[16] The mixture was stirred on a test tube mixer and allowed to react for 5 min. The product was purified on a column (3.9 × 30 mm) of Chemcosorb 7-ODS-H with a linear gradient of acetonitrile from 10% to 30% over a period of 15 min in 20 mM phosphate buffer (pH 3.0), at flow rate of 0.8 ml/min. Fractions of the highest radioactivity (retention time: 13.5 min) were used as the labelled antigen in HA-RIA.

Radioimmunoassay (RIA)——The standard diluent (SD) used was 10 mM phosphate buffer (pH 7.40), containing 0.5% bovine serum albumin (BSA), 0.025 M ethylenediaminetetraacetic acid (EDTA) and 0.15 M NaCl. The mixture in each assay tube consisted of SD (0.5 ml), peptide or tissue extract (0.1 ml), diluted antiserum (0.1 ml) and labelled antigen (0.1 ml, approximately 10000 cpm). The mixture was incubated at 4 °C overnight, and 0.5% normal rabbit serum (0.1 ml), 10% goat anti-rabbit γ-globulin serum (0.1 ml) and 30% polyethyleneglycol[19] in BSA free SD were added. After an additional incubation for 1 h at 4 °C, the mixture was centrifuged at 3000 rpm at 4 °C. The supernatant was removed and the radioactivity in the tube was counted.

Tissue Extraction——Rat whole intestine and whole brain were diced, immersed in a small amount of 2 M AcOH and then plunged into a boiling water bath for 5 min. The mixture was cooled and homogenized in a ten fold excess of ice-cold 2 M AcOH. A supernatant was obtained by centrifugation at 30000 × g for 20 min, and the precipitate was re-extracted in the same manner. The combined supernatant was washed with three portions of $CHCl_3$ and lyophilized.

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Stereoselective Synthesis of 26,27-Bisnorbrassinolide

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26,27-Bisnorbrassinolide was synthesized from pregnenolone by employing a stereoselective reduction of the 5-ylidenetetronate derivative as a key step.

Keywords——26,27-bisnorbrassinolide; plant growth promoter; 5-ylidenetetronate; stereoselective reduction; poly-hydroxy steroid; steroid side-chain

Recently, we have developed[1] a novel method for the construction of a poly-hydroxy steroid side-chain, and utilized it in the synthesis of plant growth promoters, brassinolide and (22*S*, 23*S*, 24*R*)-22,23,24-epibrassinolide. The key feature in the above synthesis involved a stereoselective reduction of the 5-ylidenetetronate to control the stereochemistry at the C-20, C-22, C-23, and C-24 positions simultaneously in one step.

We wish to report here a further application of the above synthetic strategy to the synthesis of 26,27-bisnorbrassinolide, which was shown[2] to exhibit almost the same activity as brassinolide.

Chelation-controlled addition of the dianion of 3-methyltetronic acid (**2**) to 6β-methoxy-3α,5-cyclopregnan-20-one (**1**) in tetrahydrofuran (THF) afforded the adducts (**3** and **6**), which were then converted to the corresponding methoxymethyl ethers (**4** and **7**) in 74% and 12% yields, respectively, from **1**. Dehydration[3] of the tertiary alcohol of the major product (**4**) was achieved *via* the corresponding trifluoroacetate (**5**) by treatment with trifluoroacetic anhydride, triethylamine, and 4-pyrrolidinopyridine in dichloromethane, and subsequently with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in refluxing benzene, to provide the (20*Z*)-olefin (**8**) in 80% yield together with the (20*E*)-isomer (**9**) in 7% yield, as described before.[1] With the requisite starting material in hand, the desired (20*Z*)-olefin (**8**) was subjected to stereoselective reduction over rhodium–alumina in ethyl acetate under medium pressure (7.0 atm) of hydrogen to furnish the lactone (**10**) as a sole product in 92% yield. We expected[1] that the hydrogen would attack from the back side of the steroid nucleus for steric reasons, as shown in Fig. 1, and the stereochemistry of **10** was therefore assumed to be 20*S*, 22*R*, 23*R* and 25*S*.

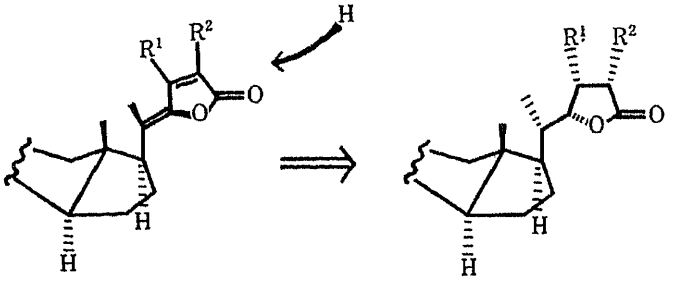
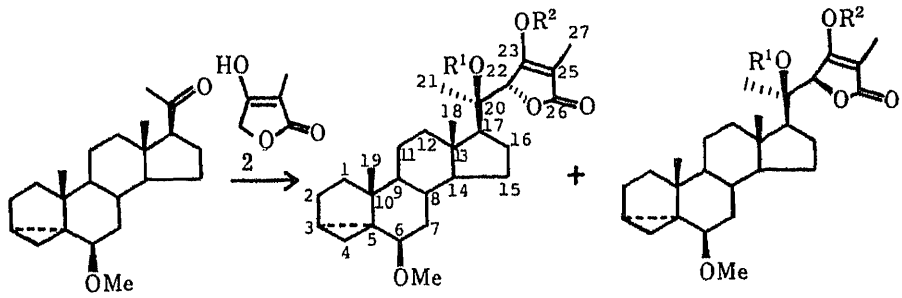


Fig. 1

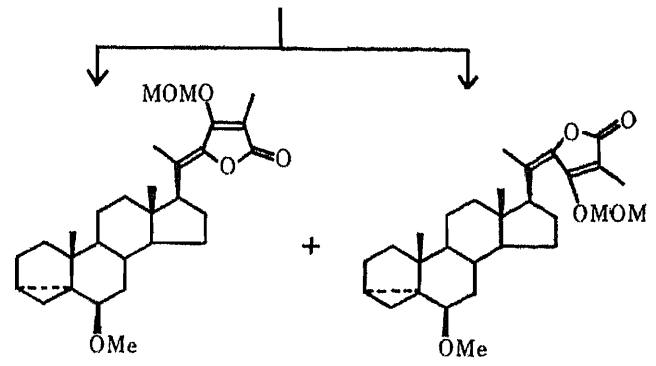
Lithium aluminum hydride reduction of **10** in THF afforded the alcohol (**11**), whose primary alcohol moiety was further converted into the corresponding mesylate (**12**) by treatment with mesyl chloride and triethylamine in dichloromethane. Reduction of **12** with



1

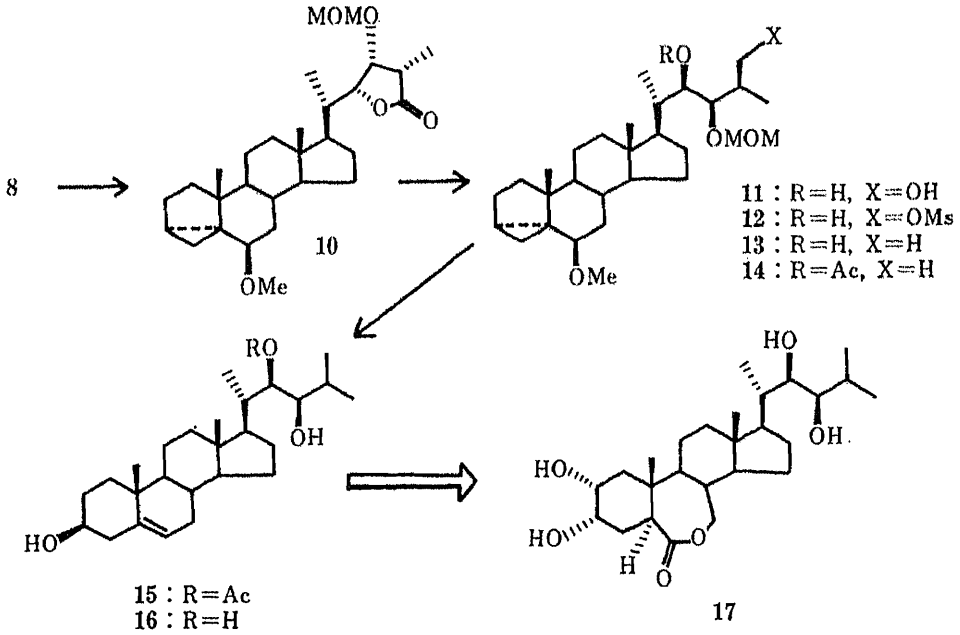
3 : R^1=H, R^2=H
4 : R^1=H, R^2=MOM
5 : R^1=$COCF_3$, R^2=MOM

6 : R^1=H, R^2=H
7 : R^1=H, R^2=MOM



8 9

Chart 1



11 : R=H, X=OH
12 : R=H, X=OMs
13 : R=H, X=H
14 : R=Ac, X=H

15 : R=Ac
16 : R=H

17

Chart 2

lithium aluminum hydride in ether gave the alcohol (**13**), which was transformed into the acetate (**14**) in 83% yield from **10**.

Finally, treatment of **14** with *p*-toluenesulfonic acid in dioxane–water afforded the olefin (**15**), which was saponified with 5% methanolic potassium hydroxide to yield the triol (**16**) in 84% yield from **14**. Since the conversion of **16** into 26,27-bisnorbrassinolide (**17**) has already been achieved by Ikekawa *et al.*,[2] the present synthesis constitutes a formal synthesis.

Thus, we were able to accomplish the stereoselective preparation of **17**. This synthetic strategy should be applicable to other important poly-hydroxy steroids.

Experimental

Infrared (IR) spectra were taken on a Hitachi 260-10 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded for solutions in $CDCl_3$ on JEOL JNM FX-100 and JNM-GX-400 spectrometers with tetramethylsilane as an internal standard. Low- and high-resolution electron impact mass spectra (MS) were taken on a JEOL JMS-D-300 spectrometer. Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP 181 instrument. Column chromatography was carried out on Wakogel C-200 (silica gel).

3-Methyltetronic Acid (2)——Bromine (117 g, 0.73 mol) in chloroform (100 ml) was added to a stirred solution of ethyl α-methylacetoacetate (100 g, 0.69 mol) in chloroform (350 ml) at 0 °C, and the reaction mixture was further stirred for 1 h at room temperature. Evaporation of the solvent gave the residue, which was heated for 2 h at 130 °C. After cooling, the solid residue was washed with hexane and recrystallized from methanol to give the tetronic acid (**2**) (54 g, 68%) as colorless needles, mp 189—190 °C (MeOH) (lit.,[4] 186—190 °C, lit.,[5] 190—191 °C). The spectroscopic data were identical with those reported.[5]

(20*R*,22*R*)-20-Hydroxy-23-methoxymethoxy-6β-methoxy-3α,5-cyclo-5α-24-norcholest-23-eno-26,22-lactone (4) and the (20*R*,22*S*)-Isomer (7)——A solution of 6β-methoxy-3α,5-cyclopregnan-20-one (**1**) (6.0 g, 18.2 mmol) in anhydrous THF (80 ml) was added to a stirred solution of the dianion [prepared from 3-methyltetronic acid (**2**) (10.0 g, 87.7 mmol) in anhydrous THF (80 ml) and lithium diisopropylamide (175.4 mmol) in anhydrous THF (80 ml)] at −78 °C under a current of nitrogen, and the reaction mixture was then stirred for 1 h at the same temperature. After quenching with aqueous ammonium chloride solution (50 ml), the mixture was extracted with ethyl acetate. The extract was washed with aqueous sodium bicarbonate solution and brine, and dried over Na_2SO_4. Evaporation of the solvent gave a pale yellow solid (8.05 g), whose solution in dry *N*,*N*-dimethylformamide (100 ml) was treated with potassium carbonate (3.00 g, 21.7 mmol) for 2 h at 70 °C. Then chloromethyl methyl ether (1.61 ml, 19.94 mmol) was added at 50 °C. The reaction mixture was stirred for 10 min at the same temperature and diluted with ethyl acetate (200 ml). The organic layer was washed with aqueous potassium hydrogen sulfate solution and brine, and dried over Na_2SO_4. Evaporation of the solvent gave two products, which were separated by chromatography on silica gel (200 g) using benzene as the eluant to give the (20*R*,22*R*)-compound (**4**) (less polar; 6.53 g, 74%) as colorless prisms, mp 131.5—133 °C (MeOH); $[\alpha]_D^{22}$ +20.08° (*c*=0.87, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1660. ^{1}H-NMR (100 MHz) δ: 0.93 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.20 (3H, s, 21-H_3), 1.93 (3H, d, *J*=1 Hz, 27-H_3), 2.76 (1H, t, *J*=2.5 Hz, 6-H), 3.32 (3H, s, 6-OMe), 3.55 (3H, s, OCH_2OCH_3), 4.57 (1H, d, *J*=1 Hz, 22-H), 5.29 and 5.37 (each 1H, each d, *J*=6 Hz, OCH_2OCH_3). MS *m/z*: 488 (M^+). *Anal.* Calcd for $C_{29}H_{44}O_6$: C, 71.28; H, 9.08. Found: C, 71.28; H, 9.27, and the (20*R*,22*S*)-compound (**7**) (more polar; 1.08 g, 12%) as a colorless amorphous powder: $[\alpha]_D^{23}$ +18.18° (*c*=1.11, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1660. ^{1}H-NMR (100 MHz) δ: 0.93 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.19 (3H, s, 21-H_3), 1.94 (3H, d, *J*=1 Hz, 27-H_3), 2.76 (1H, t, *J*=2.5 Hz, 6-H), 3.33 (3H, s, 6-OMe), 3.56 (3H, s, OCH_2OCH_3), 4.59 (1H, d, *J*=1 Hz, 22-H), 5.23 and 5.34 (each 1H, each d, *J*=6 Hz, OCH_2OCH_3). MS *m/z*: 488 (M^+). High-resolution MS *m/z*: Calcd for $C_{29}H_{44}O_6$: 488.3135. Found: 488.3104.

(20*Z*)-23-Methoxymethoxy-6β-methoxy-3α,5-cyclo-5α-24-norcholest-20(22),23-dieno-26,22-lactone (8) and the (20*E*)-Isomer (9)——Trifluoroacetic anhydride (5.65 ml, 40.0 mmol) was added dropwise to a stirred solution of the alcohol (**4**) (6.50 g, 13.3 mmol), triethylamine (5.55 ml, 40.0 mmol) and 4-pyrrolidinopyridine (590 mg, 4.0 mmol) in anhydrous dichloromethane (100 ml) at room temperature under a current of nitrogen, then the reaction mixture was stirred for 1 h and poured into water (50 ml). Isolation of the product by extraction with ethyl acetate gave the trifluoroacetate (**5**) (7.25 g, 93%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1780, 1750, 1660. ^{1}H-NMR (100 MHz) δ: 0.87 (3H, s, 18-H_3), 1.01 (3H, 2, 19-H_3), 1.73 (3H, s, 21-H_3), 1.96 (3H, d, *J*=1 Hz, 27-H_3), 2.76 (1H, t, *J*=2.5 Hz, 6-H), 3.31 (3H, s, 6-OMe), 3.52 (3H, s, OCH_2OCH_3), 5.21 (3H, br s, OCH_2OCH_3 and 22-H). MS *m/z*: 584 (M^+). High-resolution MS *m/z*: Calcd for $C_{31}H_{42}F_3O_7$: 584.2948. Found: 584.2959.

A solution of the trifluoroacetate (**5**) (7.25 g, 12.4 mmol) in anhydrous benzene (200 ml) containing DBU (2.08 ml, 13.6 mmol) was refluxed for 20 min. After cooling, the mixture was washed with aqueous potassium hydrogen sulfate solution and brine, and dried over Na_2SO_4. Evaporation of the solvent gave two products, which were separated by chromatography on silica gel (150 g) using benzene as the eluant to give the (20*Z*)-compound (**8**)

(less polar; 4.67 g, 80%) as colorless prisms, mp 174—175 °C ($MeOH$–CH_2Cl_2). $[\alpha]_D^{25}$ −134.1° ($c=1.32$, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730, 1620. ^1H-NMR (100 MHz) δ: 0.72 (3H, s, 18-H_3), 1.01 (3H, s, 19-H_3), 2.00 (3H, s, 27-H_3), 2.06 (3H, s, 21-H_3), 2.77 (1H, t, $J=2.5$ Hz, 6-H), 3.33 (3H, s, 6-OMe), 3.54 (3H, s, OCH_2OCH_3), 5.27 and 5.35 (each 1H, each d, $J=6$ Hz, OCH_2OCH_3). MS m/z: 470 (M^+). *Anal.* Calcd for $C_{29}H_{42}O_5$: C, 74.01; H, 9.00. Found: C, 73.75; H, 9.23, and the (20*E*)-compound (**9**) (0.41 g, 7%) as a colorless amorphous powder; $[\alpha]_D^{25}$ +10.0° ($c=1.09$, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730, 1620. ^1H-NMR (100 MHz) δ: 0.75 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 1.96 (3H, s, 21-H_3), 2.00 (3H, s, 25-H_3), 2.77 (1H, t, $J=2.5$ Hz, 6-H), 3.34 (3H, s, 6-OMe), 3.54 (3H, s, OCH_2OCH_3), 5.25 and 5.34 (each 1H, each d, $J=6$ Hz, OCH_2OCH_3). MS m/z: 470 (M^+). High-resolution MS m/z: Calcd for $C_{29}H_{42}O_5$: 470.3032. Found: 470.3033.

(22*R*,23*R*,25*S*)-23-Methoxymethoxy-6β-methoxy-3α,5-cyclo-5α-24-norcholestano-26,22-lactone (10)——A solution of the (20*Z*)-compound (**8**) (500 mg, 1.06 mmol) in ethyl acetate (20 ml) was hydrogenated over 5% rhodium on alumina (300 mg) for 13 h under medium pressure (7.0 atm) of hydrogen. The catalyst was filtered off and the filtrate was evaporated to afford a colorless amorphous powder (**10**) (464 mg, 92%); $[\alpha]_D^{24}$ +36.31° ($c=1.03$, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770. ^1H-NMR (400 MHz) δ: 0.76 (3H, s, 18-H_3), 1.02 (3H, s, 19-H_3), 1.14 (3H, d, $J=7$ Hz, 21-H_3 or 27 H_3), 1.24 (3H, d, $J=7$ Hz, 21-H_3 or 27-H_3), 2.70 (1H, m, 25-H), 2.78 (1H, t, $J=2.3$ Hz, 6-H), 3.33 (3H, s, 6-OMe), 3.42 (3H, s, OCH_2OCH_3), 4.31 (1H, dd, $J=8$, 4 Hz, 23-H), 4.38 (1H, dd, $J=4$, 1.5 Hz, 22-H), 4.62 and 4.68 (each 1H, each d, $J=7$ Hz, OCH_2OCH_3). MS m/z: 474 (M^+). High-resolution MS m/z: Calcd for $C_{29}H_{46}O_5$: 474.3368. Found: 474.3345.

(22*R*,23*R*,25*R*)-22,26-Dihydroxy-23-methoxymethoxy-6β-methoxy-3α,5-cyclo-5α-24-norcholestane (11)——Lithium aluminum hydride (72 mg, 1.90 mmol) was added in small portions to a stirred solution of the lactone (**10**) (300 mg, 0.63 mmol) in anhydrous THF (20 ml) under nitrogen at room temperature. The mixture was stirred for 20 min, 25% aqueous sodium hydroxide solution (3 ml) was added, and the whole was extracted with ethyl acetate. The extract was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave the diol (**11**) (300 mg, 99%) as a colorless amorphous powder: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400. ^1H-NMR (400 MHz) δ: 0.72 (3H, s, 18-H_3), 0.90 (3H, d, $J=7$ Hz, 21-H_3 or 27-H_3), 1.02 (3H, s, 19-H_3), 1.12 (3H, d, $J=7$ Hz, 21-H_3 or 27-H_3), 2.78 (1H, t, $J=2.3$ Hz, 6-H), 3.33 (3H, s, 6-OMe), 3.45 (3H, s, OCH_2OCH_3), 3.56—3.78 (4H, m, 22-H, 23-H, and 26-H_2), 4.66 and 4.81 (each 1H, each d, $J=7$ Hz, OCH_2OCH_3). MS m/z: 463 (M^+-15). High-resolution MS m/z: Calcd for $C_{28}H_{47}O_5$: 463.3421. Found: 463.3420.

(22*R*,23*R*,25*S*)-22-Acetoxy-23-methoxymethoxy-6β-methoxy-3α,5-cyclo-5α-24-norcholestane (14)——Methanesulfonyl chloride (0.052 ml, 0.67 mmol) was added slowly to a stirred solution of the diol (**11**) (300 mg, 0.63 mmol) in anhydrous dichloromethane (10 ml) containing triethylamine (0.094 ml, 0.67 mmol) under nitrogen at 0 °C. The mixture was stirred for 10 min at 0 °C, aqueous sodium bicarbonate solution (3 ml) was added, and the product was extracted with ethyl acetate to give the mesylate (**12**) (340 mg), whose solution in anhydrous ether (15 ml) was treated with lithium aluminum hydride (116 mg, 3.14 mmol) under nitrogen for 30 min at room temperature. Next, 25% aqueous sodium hydroxide solution (2 ml) was added, and the reaction mixture was extracted with ethyl acetate. The extract was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave a white solid (**13**) (243 mg), which was acetylated with acetic anhydride (1 ml) and pyridine (5 ml) containing a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (10 mg) for 10 h at room temperature. The reaction mixture was poured into water (10 ml) and isolation of the product by extraction with ethyl acetate gave a pale yellow oil, which was purified by chromatography on silica gel (10 g) using dichloromethane containing 30% chloroform as the eluant to give the acetate (**14**) (260 mg, 84%) as a pale yellow amorphous powder: $[\alpha]_D^{23}$ +32.19° ($c=1.26$, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730. ^1H-NMR (400 MHz) δ: 0.73 (3H, s, 18-H_3), 0.94 (3H, d, $J=7$ Hz, Me), 0.96 (3H, d, $J=7$ Hz, Me), 1.01 (3H, d, $J=7$ Hz, Me), 1.02 (3H, s, 19-H_3), 2.07 (3H, s, acetyl), 2.76 (1H, t, $J=2.3$ Hz, 6-H), 3.32 (3H, s, 6-OMe), 3.37 (3H, s, OCH_2OCH_3), 3.85 (1H, t, $J=9$ Hz, 23-H), 4.55 and 4.68 (each 1H, each d, $J=7$ Hz, OCH_2OCH_3), 5.13 (1H, d, $J=9$ Hz, 22-H). MS m/z: 504 (M^+). High-resolution MS m/z: Calcd for $C_{31}H_{52}O_5$: 504.3828. Found: 504.3815.

(22*R*,23*R*)-3β,22,23-Trihydroxy-24-norcholest-5-ene (16)——The acetate (**14**) (130 mg, 0.26 mmol) in dioxane (4 ml) and water (0.6 ml) was treated with *p*-toluenesulfonic acid (13 mg) for 1 h at 80 °C. The reaction mixture was diluted with ethyl acetate (20 ml), and the organic layer was washed with aqueous sodium bicarbonate solution and brine, then dried over Na_2SO_4. Evaporation of the solvent gave the 3β,23-diol (**15**) (100 mg), whose solution in 5% KOH–MeOH (4 ml) was refluxed for 1 h. The reaction mixture was diluted with ethyl acetate (30 ml), and the organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave a white solid, which was recrystallized from methanol–ethyl acetate to give the triol (**16**) (73 mg, 84%) as colorless leaflets, mp 218—220 °C (lit.,[2] 219—221 °C). The spectroscopic data were identical with those reported.[2]

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Studies on Peptides. CLI.[1,2] Syntheses of Cystine-Peptides by Oxidation of S-Protected Cysteine-Peptides with Thallium(III) Trifluoroacetate

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Thallium(III) trifluoroacetate, a mild oxidant with a soft-acid character, was found to cleave various S-protecting groups of cysteine in trifluoroacetic acid, with spontaneous formation of cystine. Except for unmasked Trp and Met, other amino acids, including His and Tyr, remained intact in the presence of this oxidant. The usefulness of this oxidant for intramolecular disulfide bond-forming reactions was demonstrated by direct conversion of three model S-protected cysteine-peptides into cystine-peptides, *i.e.*, oxytocin, urotensin II and human calcitonin gene-related peptide.

Keywords——thallium trifluoroacetate; soft-acid metal; intramolecular disulfide bond-forming reaction; cystine-peptide synthesis; S-*p*-methoxybenzylcysteine; S-1-adamantylcysteine; S-acetamidomethylcysteine; oxytocin; urotensin II; calcitonin gene-related peptide

Recently, we completed the synthesis of a 37-residue peptide[3] corresponding to the entire amino acid sequence of human calcitonin gene-related peptide (hCGRP),[4] for which a new cysteine derivative, Cys(Ad),[5] was employed. In the final step of the synthesis, the S–Ad group was cleaved by treatment with 1 M TFMSA/TFA[6] in the presence of thioanisole,[7] together with other protecting groups employed, then as usual, the deprotected peptide, after reduction with 2-mercaptoethanol, was submitted to air-oxidation to establish the disulfide bond in a highly diluted solution.

In this synthesis, we found that the S–Ad group could be cleaved by treatment with thallium (III) trifluoroacetate [$(CF_3COO)_3Tl$],[5b] a soft-acid metal which is known to have an appreciable affinity for the sulfur atom.[8] Thus, the S–Ad group was cleaved from protected hCGRP by treatment with $(CF_3COO)_3Tl$, after removing the rest of the protecting groups with TFA in the presence of thioanisole.[7b] The product thus obtained by two steps of deprotection was reduced with 2-mercaptoethanol, then submitted to air-oxidation as described above.

Hg(II) salts are known to have an ability to cleave various S-protecting groups of cysteine.[9] The resulting mercaptides have to be treated with thiols to regenerate cysteine. As in the case of Hg(II) treatment, we applied thiol treatment as described above, expecting the complete removal of the Tl salt. Later, it was found that, in contrast to Hg(II) treatment, the product formed by treatment of Z(OMe)–Cys(Ad)–OH with $(CF_3COO)_3Tl$ in TFA is cystine, not cysteine. The results indicated that $(CF_3COO)_3Tl$ acts first as a soft acid to cleave the S–Ad group, then as a mild oxidant[10] to form the disulfide bond as shown in Fig. 1. This finding suggested that S–Ad-cysteine-peptides can be directly converted to the cystine-peptides, eliminating both thiol treatment and the laborious and time-consuming air-oxidation reaction which is usually accompanied with undesired polymer formation. Before

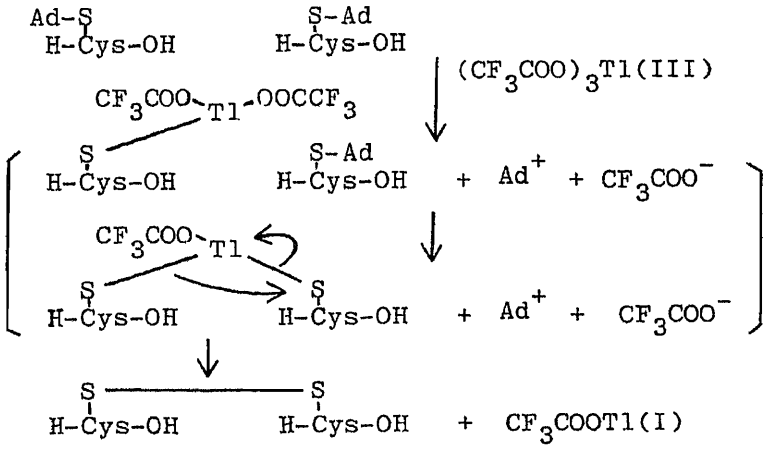


Fig. 1. Formation of Cystine by Treatment of Cys(Ad) with $(CF_3COO)_3Tl$

TABLE I. Oxidative Cleavage of Various S-Protecting Groups of Cysteine by Treatment with $(CF_3COO)_3Tl$ (0 °C, 60 min)

Derivative	Cystine formed (%)	Cysteine regenerated after reduction (%)
H-Cys(MBzl)-OH	86.7	98.5
Boc-Cys(Bu^t)-OH	80.5	96.9
Z(OMe)-Cys(Ad)-OH	83.0	89.3
Boc-Cys(Acm)-OH	81.0	95.3
H-Cys(Tri)-OH	80.2	93.6
Z(OMe)-Cys(Dbs)-OH	81.1	87.0
Boc-Cys(4-Me-Bzl)-OH	74.5[a]	89.7
H-Cys(Bzl)-OH	0[a]	0

a) A by-product, presumably the sulfoxide, was detected.

re-examination of our previous hCGRP synthesis, the responses of other S-protected cysteines to the action of this Tl salt were investigated.

In the literature, the oxidative character of iodine has been applied to convert directly Cys(Tri)-peptides and Cys(Acm)-peptides into cystine-peptides.[11] In this reaction, solvent effects have to be taken into consideration to minimize iodination at several amino acid residues, such as Tyr, His, Met and Trp. We preferred TFA as a solvent to other organic solvents, since TFA is the best solvent for free peptides, as well as protected peptides. Each Cys-derivative dissolved in TFA was treated with $(CF_3COO)_3Tl$ (1 eq) in an ice-bath for 60 min, then part of the solution was subjected to amino acid analysis. Anisole (*ca.* 2 eq) was used to trap alkyl cations. Cys(Bzl)[12] resisted the action of this reagent, but other protecting groups, including the Ad group, so far examined here (MBzl,[13] Bu^t,[14] Acm,[15] Tri,[16] and Dbs[17]) were cleaved to produce cystine as a sole product as shown in Table I. Cys(4-Me-Bzl)[18] generated cystine, but a small amount of a by-product (presumably the sulfoxide) was detected. When each cleaved sample was incubated with ethanedithiol, cysteine was regenerated quantitatively. The above results suggested that the $(CF_3COO)_3Tl$ oxidation procedure can be applied to convert not only Cys(Ad)-peptides, but also other S-protected cysteine-peptides to cystine-peptides, as well as into cysteine-peptides, if necessary.

Prior to applying this newly found disulfide-bond-forming reaction to practical peptide syntheses, the side effects of this oxidant on other functional amino acids were examined. We noticed that cystine was inert in TFA in the presence of $(CF_3COO)_3Tl$, but a small amount of

cysteic acid formed when cystine was exposed to $(CF_3COO)_3Tl$ in an aqueous solution. Thus, in practical peptide synthesis, precipitation of treated peptides with ether is recommended to remove the Tl salt, before further purification, since the Tl salt is ether-soluble. Unmasked Trp suffered modification to produce several unidentified products (recovery of Trp, 38%), but Trp(Mts)[19] remained intact after a 60 min treatment at 0 °C. Met was partially oxidized to the corresponding sulfoxide[20] (34%), but not to the sulfone. It seems worthwhile to note that His and Tyr survived unchanged after this $(CF_3COO)_3Tl$ treatment. From these model experiments, we reached the conclusion that two amino acids, Trp and Met, must be protected during the $(CF_3COO)_3Tl$ treatment.

Next, the stability of the disulfide bond of cystine under the conditions required for removal of N^{in}-protecting groups from Trp and reduction of Met(O) was examined. Recently we found that the N^{in}-Mts group of Trp could be cleaved by treatment with 1 M TMSOTf/TFA[21] in the presence of a soft base,[22] such as thioanisole[7] or diphenylsulfide[23] (PhSPh), more readily than with 1 M TFMSA/TFA,[6] together with other Bzl-type protecting groups and the N^G-Mts group from Arg.[24] Thus, as a model experiment, cystine was treated with 1 M TMSOTf/TFA or 1 M TFMSA/TFA in an ice-bath for 60 min and the effect of an added soft base was examined by measuring the recovery of cystine with an amino acid analyzer. As shown in Table II, dimethylsulfide (MeSMe) or PhSPh gave a much better result than thioanisole. Of these, MeSMe[25] (recommended for the removal of Bzl-protecting groups by TFMSA/TFA) was found not to be effective enough to assist the cleaving reaction of Trp(Mts), as well as Arg(Mts), in either 1 M TMSOTf/TFA or 1 M TFMSA/TFA treatment, as shown in Table III. Thus, the conclusion we reached here is that Trp-containing cystine-peptides can be obtained by $(CF_3COO)_3Tl$ treatment of Trp(Mts)-peptides, followed by 1 M TMSOTf/TFA treatment in the presence of PhSPh, rather than MeSMe, to remove the Mts group and other protecting groups. PhSPh is not freely soluble in TFA. Thus, a concentration

Table II. Stability of Cystine under Treatment with 1 M TMSOTf/TFA or 1 M TFMSA/TFA in the Presence of Sulfur Compounds

Reagent (0 °C, 60 min)	Cystine recovered (%)	
	1 M TFMSA/TFA	1 M TMSOTf/TFA
None	93.8	83.8
Thioanisole[a]	64.2	64.7
MeSMe[a]	92.2	86.9
PhSPh[b]	80.4	85.8

a) To a concentration of 1 M. *b*) To a concentration of 0.5 M.

Table III. Effects of Sulfur Compounds on Deprotection of Trp(Mts), Arg(Mts) and Asp(OBzl)

Additive	Deprotecting reagent (0 °C, 60 min)	Amino acid regenerated (%) from		
		Trp(Mts)	Arg(Mts)	Asp(OBzl)
PhSPh[a]	1 M TMSOTf/TFA	100.0	97.0	100.0
	1 M TFMSA/TFA	76.3	81.3	97.2
MeSMe[b]	1 M TMSOTf/TFA	34.0	22.1	98.3
	1 M TFMSA/TFA	16.7	20.1	81.4

a) To a concentration of 0.5 M. *b*) To a concentration of 1 M.

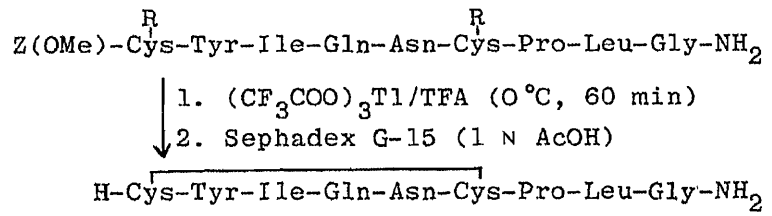
of 0.5 M PhSPh in TFA was judged to be suitable for practical use.

Next, selective deoxygenation of Met(O) in the presence of cystine was examined. It is a difficult problem to reduce Met(O) without affecting the disulfide bond, since hitherto known sulfhydryl-type reducing reagents of Met(O)[26] do not fulfill our present demand. Thioanisole[27] or MeSMe[25] in TFMSA/TFA was found to reduce Met(O) partially during TFMSA/TFA treatment, but PhSPh which we selected above as a soft base was found not to exert any noticeable reducing effect on Met(O) during TMSOTf/TFA treatment, or TFMSA/TFA treatment. Apart from sulfur compound, we were interested in examining the reducing properties of ammonium iodide.[28] A mixture of Z(OMe)–Met(O)–OH and cystine in TFA was treated with ammonium iodide in an ice-bath for 60 min, then subjected to amino acid analysis. This reagent gave tolerably high recoveries of Met and cystine as shown in Table IV. When Trp was added to this mixture, its recovery was *ca.* 76%. Thus, for the synthesis of Met- and Trp-containing cystine-peptides, further investigation seems to be required to find more suitable conditions.

After these model experiments, the usefulness of $(CF_3COO)_3Tl$ for intramolecular disulfide bond-forming reactions was examined by preparing two model peptides, oxytocin[29] and urotensin II.[30] In addition, hCGRP was re-synthesized by the present method. Each product was compared with the respective authentic samples by high-performance liquid chromatography (HPLC).

TABLE IV. Reduction of Met(O) in the Presence of Cystine and Tryptophan

Reagent (20 eq) (0 °C, 60 min)	Amino acid recovered (%)		
	Met	Cys	Trp
NH_4I	89.7	90.2	75.8



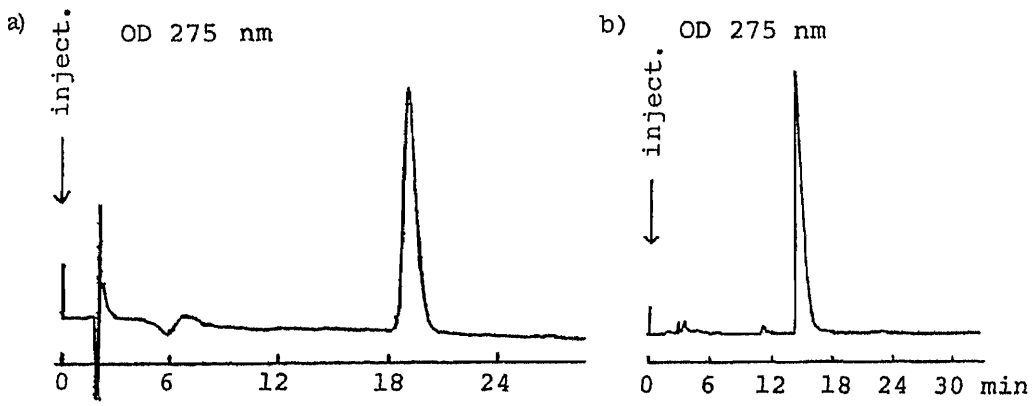


Fig. 2. HPLC of Crude Oxytocin Prepared by $(CF_3COO)_3Tl$ Oxidation

a) From the R = MBzl derivative. b) From the R = Acm derivative.

Oxytocin was prepared alternatively with Cys(MBzl) or Cys(Acm). First, when Z(OMe)–Cys(MBzl)–Tyr–Ile–Gln–Asn–Cys(MBzl)–Pro–Leu–Gly–NH_2 in TFA was treated with $(CF_3COO)_3Tl$ in an ice-bath for 60 min, oxytocin was directly obtained as a main product. The crude sample obtained after gel-filtration was examined by HPLC in comparison with an authentic sample of oxytocin. As shown in Fig. 2a, a product possessing a retention time identical with that of the authentic sample was obtained directly, without the laborious air-oxidation reaction. A similar result was obtained when the Cys(Acm) derivative, Z(OMe)–Cys(Acm)–Tyr–Ile–Gln–Asn–Cys(Acm)–Pro–Leu–Gly–NH_2, in TFA was treated with $(CF_3COO)_3Tl$, as can be seen from the HPLC elution pattern of its gel-filtered product (Fig. 2b).

Next, as an example of a Trp-containing peptide, a dodecapeptide corresponding to the entire amino acid sequence of urotensin II, a caudal neurosecretory hormone of the toleost fish, was prepared. In our previous synthesis,[31] Trp was not protected. Thus, protected urotensin II was re-synthesized using Trp(Mts). Z–Ala–Gly–Thr–Ala–Asp(OBzl)–Cys(MBzl)–Phe–Trp(Mts)–Lys(Z)–Tyr–Cys(MBzl)–Val–OBzl thus obtained was treated with $(CF_3COO)_3Tl$ in TFA as described above, then with 1 M TMSOTf/TFA in the presence of PhSPh in an ice-bath for 120 min, a longer time than in the model experiments, to ensure complete deprotection. The crude product obtained after gel-filtration was examined by HPLC. As shown in Fig. 3, a product possessing a retention time identical with that of an authentic sample of synthetic urotensin II emerged from the column as a main component. The desired product was isolated by preparative HPLC in 34% yield, while the yield of the authentic sample prepared by the usual air-oxidation procedure was 16%.

hCGRP was re-synthesized by using the present method. Protected hCGRP was first treated with $(CF_3COO)_3Tl$ in TFA to form the disulfide bond and then with 1 M TMSOTf/TFA in the presence of PhSPh as stated above to remove other protecting groups (Mts from Arg, Bzl from Ser and Z from Lys). The product was purified by gel-filtration, followed by ion-exchange chromatography on CM-Trisacryl. The elution pattern of the CM-

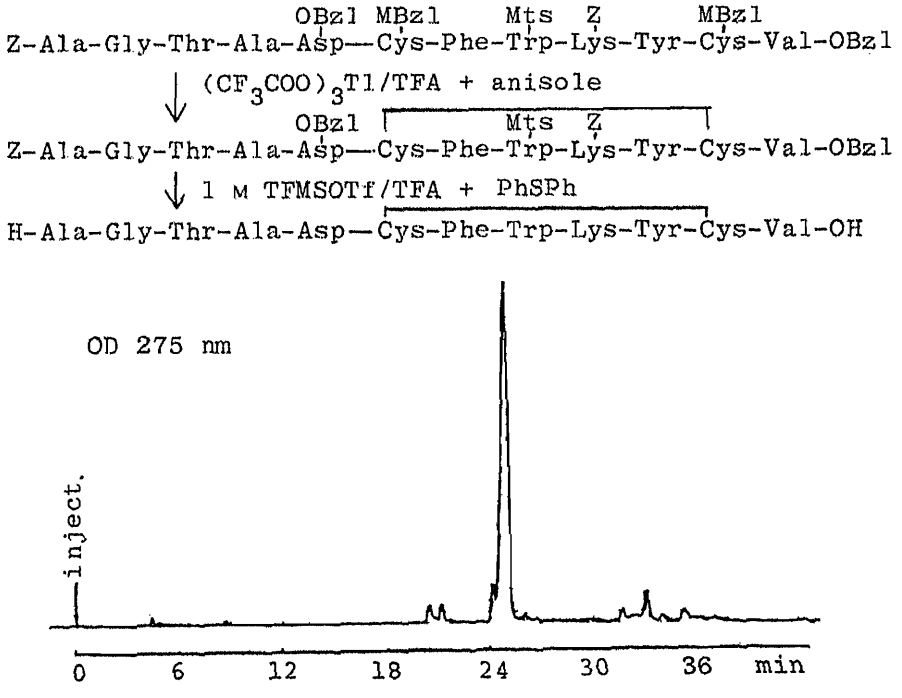


Fig. 3. HPLC of Crude Synthetic Urotensin II Prepared by $(CF_3COO)_3Tl$ Oxidation, Followed by TMSOTf/TFA Deprotection

Boc-Ala-Cys(Ad)-Asp-Thr-Ala-Thr-Cys(Ad)-Val-Thr-His-Arg(Mts)-Leu-Ala-Gly-Leu-Leu-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH_2

↓ 1. $(CF_3COO)_3Tl$/TFA + anisole
↓ 2. TMSOTf/TFA + PhSPh + *m*-cresol

H-Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2

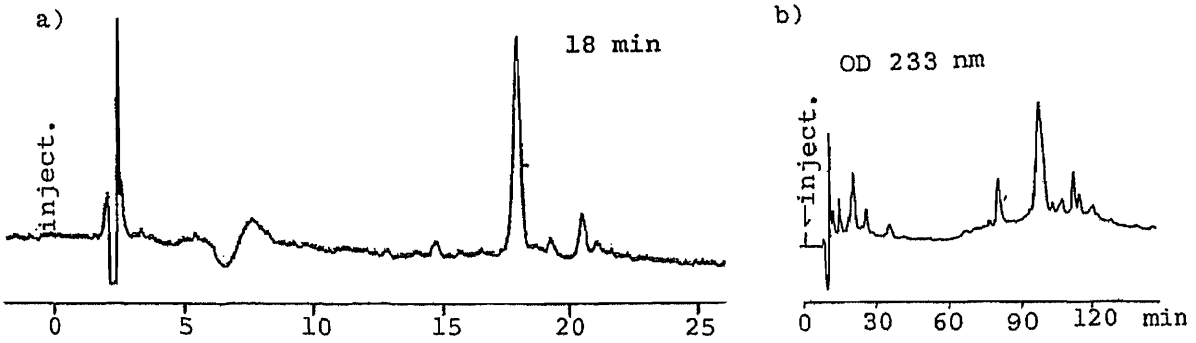


Fig. 4. HPLC of CM-Purified hCGRP

a) Sample obtained by $(CF_3COO)_3Tl$ oxidation method [on Nucleosil $5C_{18}$ (0.4 × 15 cm), flow rate 1 ml/min].
b) Sample obtained by air-oxidation method[3] [on Nucleosil $5C_{18}$ (1 × 25 cm), flow rate 2 ml/min].

purified product is shown in Fig. 4a, in comparison with that of the CM-purified product obtained by the air-oxidation procedure (Fig. 4b).[3] It can be judged that the product obtained by the present method possessed much higher purity than the previous one. After HPLC purification, the product was obtained in a somewhat better yield (11%) than before (8%).

No thallium contamination was detected in the synthetic peptides by X-ray energy spectroscopy. As described earlier, one of the attractive features on this direct oxidative disulfide bond-forming reaction is that the reaction can be performed in TFA without any solubility problem. A new route has thus been opened to synthesize peptides containing one disulfide bond without laborious and time-consuming air-oxidation.We intend to discuss some remaining problems involved in the syntheses of Met-containing cystine-peptides in a separate paper.

Experimental

Amino acid analysis and HPLC were conducted with a Hitachi 835-02 analyzer and a Waters 204 compact model, respectively. Rotation and ultraviolet absorption (UV) were determined with a Union PM-101 polarimeter and a Hitachi 100-20 spectromer, respectively. Thin layer chromatography (TLC) was conducted on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH (10:5), and Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). Fast atom bombardment mass spectra (FAB-MS) were obtained on a ZAB SE instrument (VG Analytical Co., England).

Model Experiments

$(CF_3COO)_3Tl$ Treatment of S-Protected Cysteine Derivatives——A mixture of an S-protected cysteine derivative (57 μmol each) and Gly (internal standard) in TFA (140 μl) was treated with $(CF_3COO)_3Tl$ (1 eq) in the presence of anisole (*ca.* 2 eq) in an ice-bath for 60 min. An aliquot, after being diluted with H_2O containing Met (5 eq, to quench the reagent), was analyzed on an amino acid analyzer. The results of cystine formation are shown in Table I. Presumably due to low solubility of cystine in the analysis buffers, quantitative recovery of cystine was difficult to obtain in amino acid analysis.

Reduction of $(CF_3COO)_3Tl$-Treated Samples——An aliquot of each treated sample was adjusted to pH 7.5 with 5% NH_4OH, then incubated with ethanedithiol (10 eq) at 40 °C for 5 h and submitted to amino acid analysis. The results of cysteine regeneration are listed in Table I.

Oxidation of Cystine to Cysteic Acid by $(CF_3COO)_3Tl$ in an Aqueous Solution——A mixture of cystine and $(CF_3COO)_3Tl$ (1 eq) in TFA was kept in an ice-bath for 60 min. No cysteic acid was detected. The solution was diluted with H_2O (5 times), then kept at room temperature for 24 h and subjected to amino acid analysis. Formation of cysteic acid was 11%.

Stability of Amino Acids in TFA to the Action of $(CF_3COO)_3Tl$——A mixture of Met, Tyr, His, and Trp (0.2 mmol each) in TFA (5.0 ml) was treated with $(CF_3COO)_3Tl$ (1 eq) in an ice-bath for 30 min and an aliquot was subjected to amino acid analysis. Recoveries of amino acids were: Met 66.0%, Met(O) 34.0%, Tyr 97.9% (no other peak was detected); His 96.8% (no other peak was detected), Trp 38.4%.

Stability of Cystine during 1 M TFMSA/TFA or 1 M TMSOTf/TFA Treatment in the Presence of Thioether Compounds——A mixture of cystine (45 μmol) and Gly (55 μmol, internal standard) was treated with 1 M TFMSA/TFA (0.45 ml) or 1 M TMSOTf/TFA (0.45 ml) in the presence of a thioether compound, thioanisole (to a concentration of 1 M) or MeSMe (to a concentration of 1 M) or PhSPh (to a concentration of 0.5 M), in an ice-bath for 60 min. Recoveries of cystine determined by an amino acid analyzer are listed in Table II.

Effects of Sulfur Compounds on the Deprotection of Trp(Mts), Arg(Mts) and Asp(OBzl)——A mixture of three amino acid derivatives (50 μmol each), Trp(Mts), Arg(Mts) and Asp(OBzl), was treated with 1 M TMSOTf/TFA (0.5 ml) or 1 M TFMSA/TFA (0.5 ml) in the presence of PhSPh (to a concentration of 0.5 M) or MeSMe (to a concentration of 1 M) in an ice-bath for 60 min. Recoveries of amino acids determined with an analyzer are listed in Table III.

Deoxygenation of Met(O) in the Presence of Cystine——A mixture of Z(OMe)-Met(O)-OH (35.3 mmol), cystine (27.4 mmol), H-Trp-OH (41.6 mmol), Gly (internal standard) and PhSPh (20 eq) in TFA (1 ml) was treated with NH_4I (20 eq, for Met(O)) in an ice-bath for 60 min. Recoveries of Met, Cystine and Trp are listed in Table IV.

Synthesis of Oxytocin

From Cys(MBzl)-Derivative——Z(OMe)-Cys(MBzl)-Tyr-Ile-Gln-Asn-Cys(MBzl)-Pro-Leu-Gly-NH_2 was prepared by the azide condensation of Z(OMe)-Cys(MBzl)-Tyr-$NHNH_2$ with a TFA-treated sample of Z(OMe)-Ile-Gln-Asn-Cys(MBzl)-Pro-Leu-Gly-NH_2, prepared in a stepwise manner by means of known amide-forming reactions; mp 250—251 °C, $[\alpha]_D^{20}$ −37.6° (c=0.6, DMSO), Rf_1 0.50. *Anal.* Calcd for $C_{68}H_{92}N_{12}O_{17}S_2 \cdot 3.5H_2O$: C, 55.30; H, 6.76; N, 11.38. Found: C, 55.23; H, 6.51; N, 11.17.

The above protected nonapeptide amide (37 mg) dissolved in TFA (1 ml) was treated with $(CF_3COO)_3Tl$ (15.5 mg, 1.1 eq) in the presence of anisole (0.1 ml) in an ice-bath for 60 min, then *n*-hexane was added to precipitate the product. *n*-Hexane was removed by decantation and the residue was treated with dry ether. The resulting powder was washed thoroughly with ether and dissolved in 1 N AcOH. The solution was applied to a column of Sephadex G-15 (1.6 × 103 cm), which was eluted with the same solvent. The desired fractions (4 ml each, tube Nos. 22—30, monitored by UV absorption measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a powder; yield 12 mg (45%). The HPLC pattern of the product obtained at this stage is shown in Fig. 2a. A mixture of the synthetic product and an authentic sample of oxytocin (10 μg each) emerged from a Nucleosil $5C_{18}$ (0.4 × 15 cm) column as a single peak (retention time 19 min), on isocratic elution with 16% MeCN in 0.1% TFA. For characterization, the gel-filtered sample was purified by HPLC on a TSK-GEL 410 KG column (2.45 × 30 cm) with isocratic elution using 23% MeCN in 0.1% TFA aq.; yield 7.2 mg (27%), $[\alpha]_D^{20}$ −25.2° (c=0.2, H_2O) (lit.[29] −26.2° in H_2O). Amino acid ratios in a 6 N HCl hydrolysate: Cystine 0.71, Tyr 0.91, Ile 1.01, Glu 1.00, Asp 1.02, Pro 0.96, Leu 1.00, Gly 1.00 (recovery of Gly, 79%).

From Cys(Acm)-Derivative——Boc-Cys(Acm)-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 was similarly synthesized. mp 228—230 °C, $[\alpha]_D^{19}$ −30.5° (c=0.5, DMSO), Rf_1 0.21. *Anal.* Calcd for $C_{54}H_{86}N_{14}O_{16}S_2 \cdot 0.5H_2O$: C, 51.45; H, 6.96; N, 15.56. Found: C, 51.40; H, 7.00; N, 15.27.

The protected nonapeptide obtained above (50 mg) in TFA (5.0 ml) was treated with $(CF_3COO)_3Tl$ (25.8 mg, 1.1 eq) in the presence of anisole (50 μl) and the product precipitated with ether was purified by gel-filtration on Sephadex G-15 (yield 15.1 mg, 39%), followed by HPLC as described above; yield 8.3 mg (22%). A mixture of the synthetic peptide and an authentic sample of oxytocin (10 μg each) emerged from a Vydac $5C_{18}$ (0.46 × 25 cm) column as a single peak (retention time, 14 min) on isocratic elution with 16% MeCN.

Synthesis of Urotensin II

Boc-Trp(Mts)-Lys(Z)-Tyr-Cys(MBzl)-Val-OBzl——Boc-Trp(Mts)-OSu was allowed to react with a TFA-treated sample of Z(OMe)-Lys(Z)-Tyr-Cys(MBzl)-Val-OBzl[31] in DMF in the presence of NMM until the solution became negative to the ninhydrin test. The solvent was removed by evaporation and the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O (washing procedure) and precipitated from DMF with isopropyl alcohol; yield 66%, mp 129—130 °C, $[\alpha]_D^{20}$ −27.1° (c=0.6, DMF), Rf_1 0.83. *Anal.* Calcd for $C_{71}H_{85}N_7O_{14}S_2 \cdot H_2O$: C, 63.51; H, 6.53; N, 7.30. Found: C, 63.48; H, 6.49; N, 7.46.

Boc-Cys(MBzl)-Phe-Trp(Mts)-Lys(Z)-Tyr-Cys(MBzl)-Val-OBzl——This protected heptapeptide ester was prepared by the azide condensation of Boc-Cys(MBzl)-Phe-$NHNH_2$ with a TFA-treated sample of the above

pentapeptide ester and purified by means of the above washing procedure, followed by precipitation from MeOH; yield 78%, mp 192—193 °C, $[\alpha]_D^{20}$ −26.0° (c=0.5, DMF), Rf_1 0.81. *Anal.* Calcd for $C_{91}H_{107}N_9O_{17}S_3 \cdot H_2O$: C, 63.79; H, 6.41; N, 7.36. Found: C, 63.79; H, 6.33; N, 7.38.

Z(OMe)–Asp(OBzl)–Cys(MBzl)–Phe–Trp(Mts)–Lys(Z)–Tyr–Cys(MBzl)–Val–OBzl——This protected octapeptide ester was prepared by reaction of Z(OMe)–Asp(OBzl)–OSu and a TFA-treated sample of the above protected heptapeptide ester and purified by means of the washing procedure, followed by precipitation from DMF with MeOH; yield 65%, mp 212—213 °C, $[\alpha]_D^{20}$ −59.6° (c=0.2, DMF), Rf_2 0.79. *Anal.* Calcd for $C_{106}H_{118}N_{10}O_{21}S_3 \cdot H_2O$: C, 64.22; H, 6.10; N, 7.07. Found: C, 64.18; H, 6.06; N, 7.21.

Z–Ala–Gly–Thr–Ala–Asp(OBzl)–Cys(MBzl)–Phe–Trp(Mts)–Lys(Z)–Tyr–Cys(MBzl)–Val–OBzl——Protected urotensin II was prepared by the azide condensation of Z–Ala–Gly–Thr–Ala–$NHNH_2$ with a TFA-treated sample of the above protected octapeptide ester and purified by means of the washing procedure followed by precipitation from DMF with MeOH; yield 77%, mp 274—276 °C, $[\alpha]_D^{20}$ −40.8° (c=0.3, DMF), Rf_1 0.69, Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.92, Thr 0.99, Gly 1.03, Ala 1.83, Val 0.94, Tyr 1.17, Phe 1.00, Lys 1.05 (recovery of Phe, 85%). *Anal.* Calcd for $C_{117}H_{136}N_{14}O_{25}S_3 \cdot 2H_2O$: C, 61.89; H, 6.22; N, 8.64. Found: C, 61.81; H, 6.00; N, 8.61.

Deprotection for the Synthesis of Urotensin II——Protected urotensin II (50 mg) in TFA (5.0 ml) was treated with $(CF_3COO)_3Tl$ (14.6 mg, 1.2 eq) in the presence of anisole (50 μl) in an ice-bath for 60 min, then TFA was removed by evaporation *in vacuo* and dry ether was added. The resulting powder was collected by centrifugation, then treated with 1 M TMSOTf/TFA (4 ml) in the presence of PhSPh (0.79 ml) and *m*-cresol (0.25 ml) in an ice-bath for 120 min. Finally isopropyl ether was added. The resulting powder was collected by centrifugation, washed with isopropyl ether and dissolved in H_2O (5 ml). The pH of the ice-chilled solution was adjusted to 7.5 with 5% NH_4OH and after 10 min, to 3 with AcOH. The solution was applied to a column of Sephadex G-25 (2.0 × 137 cm), which was eluted with 1 N AcOH. The desired fractions (6.7 ml each, tube Nos. 60—67, monitored by UV absorption measurement at 275 nm) were combined and the solvent was removed by lyophilization. The HPLC elution pattern of this crude product was shown in Fig. 3. The product was next purified by HPLC on a Cosmosil $5C_{18}$ column with a gradient of MeCN (23 to 35%, for 20 min) in 0.1% TFA. The desired eluate (retention time, 25 min) was collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 10.4 mg (34%). $[\alpha]_D^{20}$ −59.0° (c=0.2, 1% AcOH), (lit.[31] −60.4° in 1% AcOH). FAB-MS *m/z*: 1361 $(M+H)^+$. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.02, Thr 0.92, Gly 1.01, Ala 1.96, Cys 0.86, Val 1.00, Tyr 1.00, Phe 1.02, Lys 1.05 (recovery of Val, 78%). A mixture of the samples obtained by the present method and the previous method emerged from a Nucleosil $5C_{18}$ column as a single peak (retention time, 25 min), when eluted with the same gradient employed above.

Re-synthesis of hCGRP

Protected hCGRP (75 mg) in TFA (7.5 ml) was treated with $(CF_3COO)_3Tl$ (9.7 mg, 1.2 eq) in the presence of anisole (75 μl) in an ice-bath for 60 min, then TFA was removed by evaporation *in vacuo* and dry ether was added. The resulting powder was collected by centrifugation, and treated with 1 M TMSOTf/TFA (5.0 ml) in the presence of PhSPh (1.2 ml) and *m*-cresol (0.37 ml) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by centrifugation and dissolved in H_2O (5 ml). The pH of the ice-chilled solution was adjusted to 7.5 with 5% NH_4OH and after 10 min, to 3.0 with AcOH. The solution was gel-filtered on Sephadex G-25 using 1 N AcOH as an eluant. The desired fractions (6.7 ml each, tube Nos. 43—55, monitored by means of the Folin–Lowry test at 750 nm) were combined and the solvent was removed by lyophilization; yield 41 mg (73%). The product was next purified by ion-exchange chromatography on a CM-Trisacryl column (2 × 5 cm) with a gradient of NaCl (0 to 0.2 M) in pH 6.5, 0.01 M $AcONH_4$ buffer. The desired fractions (5.8 ml each, tube Nos. 53—62, monitored by means of the Folin–Lowry test at 750 nm) were combined and the solvent was removed by lyophilization. The residue was desalted by gel-filtration on Sephadex G-15 using 1 N AcOH as an eluant as stated above. Lyophilization of the desired fractions gave a fluffy powder; yield 12 mg (30%). This CM-purified product was further purified by HPLC on a Nucleosil $5C_{18}$ column with a gradient of MeCN (25—40%, 30 min) in 0.1% TFA aq. at a flow rate of 1.0 ml/min. Its HPLC pattern is shown in Fig. 4. After a further run of HPLC, the desired eluate (retention time, 18 min) was taken and the solvent was removed by lyophilization to give a white fluffy powder; yield 3.0 mg (52%), overall yield was 11%. $[\alpha]_D^{20}$ −81.1° (c=0.1, 0.5 N AcOH) (lit.[3] −84.4° in 0.5 N AcOH), FAB-MS *m/z*: 3789 $(M+H)^+$. Amino acid ratios in a 6 N HCl hydrolysate (numbers in parentheses are theoretical): Asp. 4.21 (4), Thr 3.75 (4), Ser 3.01 (3), Pro 0.97 (1), Gly 4.09 (4), Ala 4.08 (4), Cys 0.69 (1), Val 4.29 (5), Leu 3.14 (3), Phe 2.00 (2), Lys 2.05 (2), His 0.96 (1), Arg 2.09 (2), (recovery of Phe was 80%, low recovery of Val was due to incomplete hydrolysis of the Val–Val linkage).

The product obtained here was identical in terms of Rf values on TLC (Rf_3 0.39) and retention time (18 min) on HPLC with the product obtained by the previous air-oxidation procedure under the conditions stated above.

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Chemical Transformation of Protoberberines. XIII.[1] A Novel and Efficient Synthesis of Antitumor Benzo[*c*]phenanthridine Alkaloids, Nitidine and Fagaronine[2]

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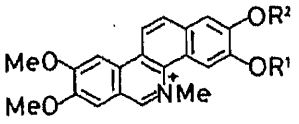
An efficient synthesis of nitidine (**1a**),and fagaronine (**1c**), antitumor 2,3,8,9-tetraoxygenated benzo[*c*]phenanthridine alkaloids, has been achieved *via* regioselective C_6–N bond cleavage, followed by consecutive oxy-functionalization and recyclization between the C_6 and C_{13} positions of the starting protoberberines, pseudoberberine (**3a**) and *O*-benzyldehydrodiscretine (**3b**), respectively.

Keywords——2,3,8,9-tetraoxygenated benzo[*c*]phenanthridine; nitidine; fagaronine; antitumor alkaloid; protoberberine; pseudoberberine; *O*-benzyldehydrodiscretine; thallium trinitrate

More than eighty benzo[*c*]phenanthridine alkaloids have so far been isolated from Papaveraceae and Rutaceae plants.[3] Among them, 2,3,8,9-tetraoxygenated benzo[*c*]-phenanthridine alkaloids, nitidine (**1a**) and fagaronine (**1c**), are of great interest because of their strong antileukemic activity in the leukemia L-1210 and P-388 systems in mice as well as Lewis lung carcinoma.[4–7] Much effort has been directed towards the development of a convenient synthesis of nitidine (**1a**),[7,8] and two syntheses of fagaronine (**1c**)[9] have also been reported.



nitidine: $R^1+R^2=CH_2$
fagaronine: $R^1=Me$, $R^2=H$

Fig. 1

Recently we have developed an efficient method[10,11] for synthesis of the fully aromatized benzo[*c*]phenanthridine skeleton from protoberberine by a biomimetic route[12] and succeeded in the synthesis of chelerythrine[10] and sanguilutine,[11] representative 2,3,7,8-tetraoxygenated and 2,3,7,8,10-pentaoxygenated benzo[*c*]phenanthridine alkaloids, respectively. We now describe a novel and efficient synthesis of the antitumor 2,3,8,9-tetraoxygenated benzo[*c*]phenanthridine alkaloids, nitidine (**1a**) and fagaronine (**1c**), from the corresponding protoberberines (**3a** and **3b**) by our biomimetic procedure.

Synthesis of Nitidine

Reduction of pseudoberberine (**3a**)[13,14] with lithium aluminum hydride (LAH) in dry tetrahydrofuran (THF) at room temperature gave an enamine, which was treated with dimethyl sulfate in refluxing benzene to afford the methosulfate (**4a**) (95%). The Hofmann elimination of **4a** with 25% methanolic potassium hydroxide provided the C_6–N bond-cleaved product. This labile styrene derivative was subsequently exposed to 2,3-dichloro-5,6-dicyano-

1,4-benzoquinone (DDQ) in chloroform at room temperature and then to potassium ferricyanide in the presence of 25% aqueous potassium hydroxide in refluxing methanol to furnish the stable enamide (**5a**) (41% yield from **4a**). The structure of **5a** was established by analysis of its spectral data (see Experimental section).

Introduction of an oxygen function at the terminal of the styrene moiety in **5a** was achieved by treatment with thallium (III) trinitrate (TTN)[15] in methanol at room temperature to give the acetal (**6a**) (91%). The structure of **6a** was apparent from the signals of the acetal protons at 4.83 (1H, dd, $J=6$ and 5 Hz), 3.23 (3H, s), and 3.17 ppm (3H, s) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. On treatment with 10% hydrochloric acid, the acetal (**6a**) underwent hydrolysis, cyclization, and dehydration to produce the benzo[*c*]phenanthridine, oxynitidine (**7a**) (93%). When the enamide (**5a**) was successively treated with TTN in methanol and 10% hydrochloric acid, oxynitidine (**7a**) was obtained in quantitative yield. The synthetic oxynitidine was found to be identical with an authentic sample of the alkaloid by comparison of their spectra. Since oxynitidine (**7a**) has already been converted into dihydronitidine (**8a**) and nitidine (**1a**),[8e] the present synthesis amounts to a formal synthesis of **8a** and **1a**. In addition, we have accomplished an alternative transformation of **7a** into **8a** and **1a**. Oxynitidine (**7a**) was reduced with LAH in dry THF at room temperature to give dihydronitidine (**8a**) (79%), which was finally oxidized with DDQ in the presence of 5% aqueous sodium hydroxide to yield nitidine chloride (**1a**) (88%) after treatment with concentrated hydrochloric acid. Both synthetic dihydronitidine and nitidine were proved to be identical with the corresponding authentic alkaloids by comparison of their spectra. Thus, we succeeded in an efficient synthesis of oxynitidine, dihydronitidine, and nitidine from pseudoberberine (**3a**) by our biomimetic method.

Synthesis of Fagaronine

Fagaronine (**1c**) was isolated from *Fagara zanthoxyloides* LAM.[5] in 1972 and found to possess strong antileukemic activity with low cytotoxicity.[7] Although fagaronine (**1c**) has the

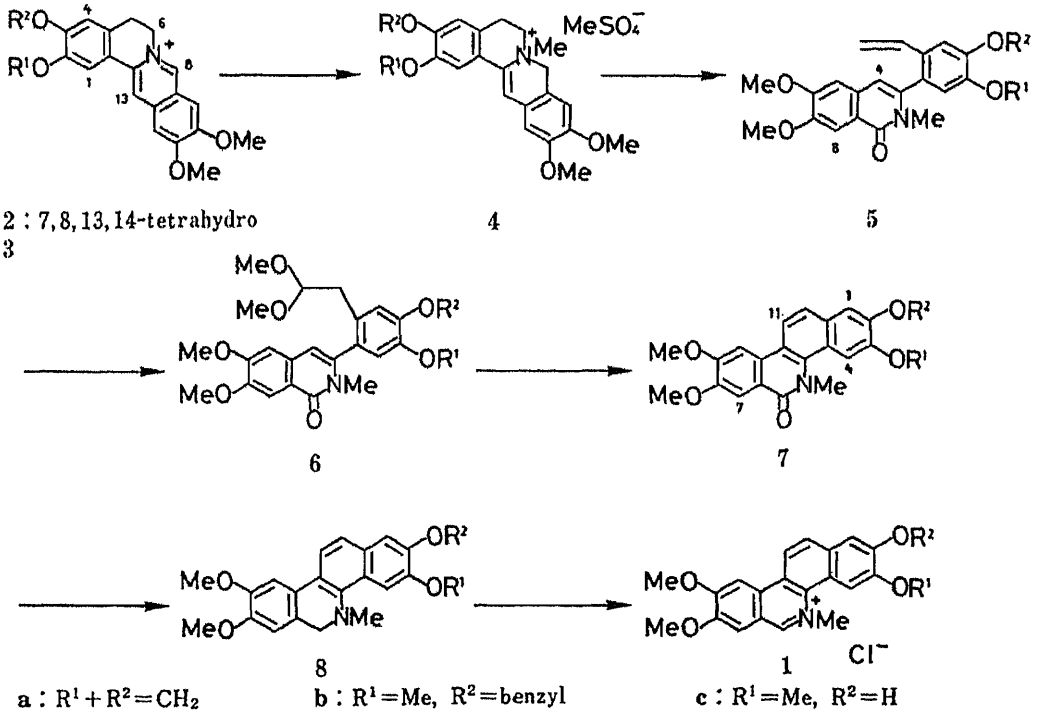


Chart 1

same oxygen substituent pattern on the aromatic rings as nitidine (**1a**), the presence of the phenolic hydroxy group at the C_2 position made the synthesis of **1c** more difficult. One of the most crucial points in the synthesis of **1c** is how to protect the hydroxy group at C_2. In the two previous syntheses of **1c**,[9] the hydroxy group was protected as an isopropyl ether instead of a common benzyl ether because debenzylation took place during the syntheses. As all steps proceed under relatively mild conditions in our biomimetic procedure, we selected the conventional benzyl group as a protecting group and therefore began with *O*-benzyldiscretine (**2b**).

O-Benzyldiscretine (**2b**)[16] was dehydrogenated with iodine in the presence of potassium acetate in refluxing ethanol to give *O*-benzyldehydrodiscretine (**3b**) (84%). This was reduced with LAH in dry THF, then treated with dimethyl sulfate in refluxing benzene to afford the methosulfate (**4b**) (81%). Exposure of **4b** to 25% methanolic potassium hydroxide effected C_6–N bond fission to provide a labile enamine, which on successive treatment with DDQ and potassium ferricyanide yielded the stable enamide (**5b**) (46%). The structure of **5b** was established by analysis of the spectral data.

The enamide (**5b**)was oxidized with TTN[15] in methanol at room temperature to give the acetal (**6b**) (92%), hydrolysis of which with 10% hydrochloric acid provided, as expected, *O*-benzyl-oxyfagaronine (**7b**) (98%). Successive treatment of the enamide (**5b**) with TTN and 10% hydrochloric acid without isolation of the intermediate acetal (**6b**) gave *O*-benzyl-oxyfagaronine (**7b**) in quantitative yield. A salient feature of **7b** was the lower field proton signal of C_{11}-H at 7.98 ppm (1H, d, J=9 Hz). Sequential reduction of **7b** with LAH in dry THF and sodium borohydride[17] in methanol at room temperature gave *O*-benzyldi-hydrofagaronine (**8b**) (*m/z*: 441 (M^+) δ 4.15 (2H, s)) (95%), dehydrogenation of which with DDQ yielded *O*-benzylfagaronine (**1b**) (δ 9.93 (1H, s)) (93%). Finally, fagaronine (**1c**) was obtained in 97% yield by debenzylation of **1b** with 6 N hydrochloric acid in ethanol under reflux. The structure of **1c** was confirmed by the spectral data. The synthetic alkaloid (**1c**) was identical with natural fagaronine, based on a comparison of their spectra. Fagaronine (**1c**) was converted into dihydrofagaronine (**8c**)[9b] (82%) by sodium borohydride reduction at room temperature.

Thus, we accomplished an efficient and convenient synthesis of nitidine (**1a**) and fagaronine (**1c**), antitumor 2,3,8,9-tetraoxygenated benzo[*c*]phenanthridine alkaloids, from the corresponding protoberberines, pseudoberberine (**3a**) and *O*-benzyldehydrodiscretine (**3b**), respectively, by a biomimetic route. In combination with the previous success in the synthesis of 2,3,7,8-[10] and 2,3,7,8,10-oxygenated[11] alkaloids, the present synthesis demonstrated the potential versatility of our biomimetic method for the preparation of all types of fully aromatized benzo[*c*]phenanthridine alkaloids including phenolic ones. The utility of our synthesis is further enhanced by the fact that the starting protoberberine alkaloids are naturally abundant and can be easily synthesized.[18]

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a JASCO A-102 spectrometer, mass spectra (MS) with a Hitachi M-80 mass spectrometer, ultraviolet (UV) spectra with a Hitachi 323 spectrometer, and ^{1}H-NMR spectra with a JEOL FX-100 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard, unless otherwise stated. Alumina (Aluminiumoxid 90, Akitivitätsstufe II-III, 70—230 mesh, Merck) was used for column chromatography. Organic extracts were dried over anhydrous Na_2SO_4.

3-Benzyloxy-5,6-dihydro-2,10,11-trimethoxydibenzo[*a*,*g*]quinolizinium Iodide (*O*-Benzyldehydrodiscretine) (3b) ——A solution of iodine (14.3 g, 5.6 mmol) in ethanol (300 ml) was added dropwise to a refluxing mixture of *O*-benzyldiscretine (**2b**) (8.1 g, 19 mmol) and potassium acetate (8.3 g, 85 mmol) in ethanol (300 ml) over a period of 15 min, and reflux was continued for 2 h. After cooling of the reaction mixture to room temperature, the precipitates were collected by filtration. Sulfur dioxide gas was passed through a suspension of the precipitates in water (200 ml)

for 1 h with stirring. The yellow precipitates were collected by filtration and dried to give **3b** (8.8 g, 84%), yellow needles, mp 277—280 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 241 (4.33), 266 (4.36), 289 (4.70), 310 sh (4.33), 341 (4.36), 378 (3.93). ^{1}H-NMR ($(CD_3)_2SO$) δ: 9.53 (1H, s, H-8), 8.87, 7.62, 7.22 (each 1H, each s, aromatic H), 7.71 (2H, s, aromatic H), 7.54—7.33 (5H, m, aromatic H), 5.20 (2H, s, benzylic H), 4.88—4.70 (2H, m, H-6), 4.08, 4.01, 3.95 (each 3H, each s, OMe × 3), 3.74—3.47 (2H, m, H-5). *Anal.* Calcd for $C_{27}H_{26}INO_4$: C, 58.38; H, 4.72; N, 2.52. Found: C, 58.42; H, 4.66; N, 2.14.

5,6,7,8-Tetrahydro-10,11-dimethoxy-7-methyl-2,3-methylenedioxydibenzo[*a*,*g*]quinolizinium Monomethylsulfate (4a)——Pseudoberberine iodide (**3a**) (4.4 g, 9.5 mmol) was added portionwise to a stirred suspension of LAH (1.5 g, 38 mmol) in dry THF (150 ml) over a period of 15 min in a stream of nitrogen at 0 °C. The suspension was stirred for 3 h at room temperature, then water was added and the whole was filtered. The filtrate was concentrated to leave the crude dihydro derivative, which was dissolved in benzene (100 ml). This solution was heated under reflux. Dimethyl sulfate (9.0 g, 71 mmol) was added dropwise to the refluxing benzene solution and refluxing was continued for 2 h. After cooling of the reaction mixture to room temperature, the resulting precipitates were collected by filtration and dried to provide **4a** (4.2 g, 95%), yellow pillars, mp 274—274.5 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 233 (4.29), 240 (4.31), 248 (4.31), 265 sh (4.06), 288 (4.01), 342 sh (4.31), 358 (4.45), 376 (4.39). ^{1}H-NMR ($(CD_3)_2SO$) δ: 7.70, 7.54 (each 1H, each s, aromatic H), 7.11 (2H, s, aromatic H), 6.93 (1H, s, olefinic H), 6.01 (2H, s, OCH_2O), 5.01, 4.78 (each 1H, AB-q, J = 14.5 Hz, H-8), 4.04 (2H, d, J = 6 Hz, H-6), 3.86, 3.83 (each 3H, each s, OMe × 2), 3.65—2.80 (2H, m, H-5), 3.33, 3.05 (each 3H, each s, $MeSO_4^-$ and NMe). *Anal.* Calcd for $C_{22}H_{25}NO_8S \cdot H_2O$: C, 54.87; H, 5.65; N, 2.91. Found: C, 54.61; H, 5.74; N, 2.61.

3-Benzyloxy-5,6,7,8-tetrahydro-2,10,11-trimethoxy-7-methyldibenzo[*a*,*g*]quinolizinium Monomethylsulfate (4b)——*O*-Benzyldehydrodiscretine (**3b**) (6.7 g, 12 mmol) was reduced with LAH (2.6 g, 68 mmol) and the resulting dihydroderivative was treated with dimethyl sulfate (10 g, 79 mmol) as described for **4a** to afford **4b** (5.4 g, 81%), yellow pillars, mp 275—280 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 233 sh (4.28), 242 (4.33), 248 (4.30), 265 (4.19), 289 (4.40), 314 (4.30), 356 (4.43), 374 (4.36). ^{1}H-NMR ($(CD_3)_2SO$) δ: 7.79, 7.18, 7.11, 7.06 (each, 1H, each s, aromatic H), 7.56—7.22 (6H, m, aromatic H and olefinic H), 5.15 (2H, s, benzylic H), 5.00, 4.80 (each 1H, AB-q, J = 14 Hz, H-8), 4.16—3.68 (2H, m, H-6), 3.89, 3.86, 3.84 (each 3H, each s, OMe × 3), 3.44—2.76 (2H, m, H-5), 3.37, 3.05 (each 3H, each s, $MeSO_4^-$ and NMe). *Anal.* Calcd for $C_{29}H_{33}NO_8S$: C, 62.68; H, 5.99; N, 2.52. Found: C, 62.57; H, 5.73; N, 2.40.

6,7-Dimethoxy-2-methyl-3-(4,5-methylenedioxy-2-vinylphenyl)isoquinolin-1(2*H*)-one (5a)——The methosulfate (**4a**) (499 mg, 1.08 mmol) was added at once to refluxing 25% methanolic potassium hydroxide (8 ml) and the mixture was heated under reflux for 10 min. The reaction mixture was poured into ice-water (20 ml) and the resulting precipitates were collected by filtration. The precipitates were dissolved in chloroform (20 ml), and a solution of DDQ (227 mg, 1.0 mmol) in chloroform (30 ml) was added dropwise. The mixture was stirred for 3 h at room temperature, chloroform was evaporated off, and the residue was dissolved in methanol (10 ml). A solution of potassium ferricyanide (1.0 g, 3.1 mmol) in 25% aqueous potassium hydroxide (20 ml) was added to the methanol solution and the reaction mixture was refluxed for 5 h then filtered. The filtrate was extracted with ethyl acetate. The extract was washed with water and brine, and concentrated to dryness. Column chromatography of the residue on alumina with ethyl acetate : hexane (1 : 1) gave **5a** (161 mg, 41%), pale yellow pillars, mp 196—197 °C (EtOH). IR ν_{max} cm^{-1}: 1645 (amide). UV λ_{max}^{EtOH} nm (log ε): 233 sh (4.61), 251 sh (4.70), 300 (4.27), 340 sh (3.81). ^{1}H-NMR δ: 7.83 (1H, s, H-8), 7.14, 6.84, 6.72 (each 1H, each s, aromatic H), 6.41 (1H, dd, J = 17.5, 11 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 6.34 (1H, s, H-4), 6.04 (2H, s, OCH_2O), 5.59 (1H, d, J = 17.5 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 5.11 (1H, d, J = 11 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 4.03, 3.98 (each 3H, each s, OMe × 2), 3.29 (3H, s, NMe). MS *m/z* (%): 365 (M^+, 100), 350 (17), 204 (31). *Anal.* Calcd for $C_{21}H_{19}NO_5$: C, 69.03; H, 5.24; N, 3.83. Found: C, 68.73; H, 5.44; N, 3.68.

3-(4-Benzyloxy-5-methoxy-2-vinylphenyl)-6,7-dimethoxy-2-methylisoquinolin-1(2*H*)-one (5b)——The Hofmann elimination of the methosulfate (**4b**) (500 mg, 0.9 mmol), followed by successive DDQ (600 mg, 2.6 mmol) and potassium ferricyanide (3.0 g, 9.1 mmol) oxidations according to the procedure described for **5a** afforded **5b** (188 mg, 46%) after chromatography on alumina with ethyl acetate : hexane (1 : 1), pale yellow pillars, mp 205—206 °C (MeOH). IR ν_{max} cm^{-1} 1640 (amide). UV λ_{max}^{MeOH} nm (log ε): 251 (4.74), 299 (4.28). ^{1}H-NMR δ: 7.84 (1H, s, H-8), 7.56—7.20 (5H, m, aromatic H), 7.20, 6.84, 6.77 (each 1H, each s, aromatic H), 6.24 (1H, dd, J = 17, 11 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 6.37 (1H, s, H-4), 5.49 (1H, dd, J = 17, 1 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 5.24 (2H, s, benzylic H), 5.09 (1H, dd, J = 11, 1 Hz, $\overset{H}{H}>=<\overset{H}{Ar}$), 4.03, 3.97, 3.89 (each 3H, each s, OMe × 3), 3.28 (3H, s, NMe). MS *m/z* (%): 457 (M^+, 100), 366 (51), 338 (92), 91 (61). *Anal.* Calcd for $C_{28}H_{27}NO_5$: C, 73.50; H, 5.95; N, 3.06. Found: C, 73.63; H, 5.91; N, 2.99.

6,7-Dimethoxy-3-[2-(2,2-dimethoxyethyl)-4,5-methylenedioxyphenyl]-2-methylisoquinolin-1(2*H*)-one (6a)——A solution of TTN trihydrate (48 mg, 0.11 mmol) in methanol (1 ml) was added dropwise to a solution of **5a** (30 mg, 0.08 mmol) in methanol (5 ml) at room temperature and the reaction mixture was stirred for 10 min, then filtered. Methylene dichloride (15 ml) was added to the filtrate and the extract was washed with saturated aqueous sodium

bicarbonate, water, and brine, dried, and concentrated to leave **6a** (32 mg, 91%) as an oil. IR ν_{max} cm^{-1}: 1640 (amide). ^{1}H-NMR δ: 7.83 (1H, s, H-8), 6.93, 6.92, 6.69 (each 1H, each s, aromatic H), 6.33 (1H, s, H-4), 6.02 (2H, s, OCH_2O), 4.38 (1H, dd, J=6, 5 Hz, $CH(OMe)_2$), 4.02, 3.98 (each 3H, each s, OMe×2), 3.31 (3H, s, NMe), 3.23, 3.17 (each 3H, each s, OMe×2), 2.81 (1H, dd, J=14.5, 6 Hz, benzylic H), 2.61 (1H, dd, J=14.5, 5 Hz, benzylic H). MS m/z (%): 427 (M^+, 7.8), 352 (4.5), 75 (100). High-resolution mass Calcd for $C_{23}H_{25}NO_7$: 427.1629. Found: 427.1615.

3-[4-Benzyloxy-5-methoxy-2-(2,2-dimethoxyethyl)-phenyl]-6,7-dimethoxy-2-methylisoquinolin-1(2*H*)-one (6b)——The enamide (**5b**) (39 mg, 0.09 mmol) was treated with TTN trihydrate (47 mg, 0.10 mmol) as described for **6a** to yield **6b** (41 mg, 92%) as an oil. IR ν_{max} cm^{-1}: 1640 (amide). ^{1}H-NMR δ: 7.83 (1H, s, H-8), 7.55—7.20 (5H, m, aromatic H), 6.98, 6.82, 6.72 (each 1H, each s, aromatic H), 6.34 (1H, s, H-4), 5.22 (2H, s, benzylic H), 4.31 [1H, dd, J=6, 5 Hz, $CH(OMe)_2$], 4.03, 3.98, 3.87 (each 3H, each s, OMe×3), 3.30 (3H, s, NMe), 3.14, 3.12 (each 3H, each s, OMe×2), 2.79 (1H, dd, J=14, 6 Hz, benzylic H), 2.60 (1H, dd, J=14, 5 Hz, benzylic H). MS m/z (%): 457 (M^+, 100), 366 (51), 338 (92), 91 (61). High-resolution mass Calcd for $C_{30}H_{33}NO_7$: 519.2235. Found: 519.2246.

8,9-Dimethoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridin-6(5*H*)-one (Oxynitidine) (7a)——A solution of **6a** (75 mg, 0.18 mmol) and 10% hydrochloric acid (1 ml) in methanol (10 ml) was refluxed for 30 min, then the methanol was evaporated off and the residue was taken up in methylene dichloride. The solution was washed with water and brine, dried, and concentrated to leave **7a** (59 mg, 93%), colorless needles, mp 284—285 °C (EtOH) (lit.[8c] 284—285 °C). IR ν_{max} cm^{-1}: 1640 (amide). UV λ_{max}^{EtOH} nm (log ε): 251 (4.65), 278 (4.80), 289 (4.84), 335 (4.30). ^{1}H-NMR δ: 7.95, 7.53 (each 1H, AB-q, J=9 Hz, H-11 and H-12), 7.91 (1H, s, H-7), 7.61, 7.56, 7.16 (each 1H, each s, aromatic H), 6.09 (2H, s, OCH_2O), 4.09, 4.05, 3.96 (each 3H, each s, OMe×2 and NMe). MS m/z (%): 363 (M^+, 100), 334 (8), 305 (9). *Anal.* Calcd for $C_{21}H_{17}NO_5$: C, 69.41; H, 4.72; N, 3.86. Found: C, 69.23; H, 4.72; N, 3.90. The product was identical with natural oxynitidine on the basis of ^{1}H-NMR, MS, and thin-layer chromatographic (TLC) comparisons.

2-Benzyloxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridin-6(5*H*)-one (*O*-Benzyl-oxyfagaronine) (7b)——The acetal (**6b**) (39 mg, 0.07 mmol) was hydrolyzed with 10% hydrochloric acid as described for **7a** to give **7b** (34 mg, 98%), colorless needles, mp 227—229 °C (MeOH). IR ν_{max} cm^{-1}: 1640 (amide). UV λ_{max}^{MeOH} nm (log ε): 230 (4.31), 253 (4.63), 267 (4.72), 277 (4.77), 288 (4.87), 320 (4.26), 333 (4.24). ^{1}H-NMR δ: 7.98 (1H, d, J=9 Hz, H-11), 7.93 (1H, s, H-7), 7.65—7.25 (7H, m, aromatic H), 7.64, 7.23 (each 1H, each s, aromatic H), 5.31 (2H, s, benzylic H), 4.10 (3H, s, OMe), 4.06 (6H, s, OMe×2), 4.04 (3H, s, NMe). MS m/z (%): 455 (M^+, 100), 364 (85), 336 (29), 306 (17), 91 (51). *Anal.* Calcd for $C_{28}H_{25}NO_5$: C, 73.83; H, 5.53; N, 3.08. Found: C, 73.83; H, 5.41; N, 3.07.

Sequential treatment of the enamide (**5b**) (293 mg, 0.64 mmol) with TTN trihydrate (313 mg, 0.71 mmol) and 10% hydrochloric acid directly afforded **7b** (291 mg, 100%).

5,6-Dihydro-8,9-dimethoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridine (Dihydronitidine) (8a)——LAH (105 mg, 2.8 mmol) was added portionwise to a stirred solution of **7a** (46 mg, 0.13 mmol) in dry THF (20 ml) in a stream of nitrogen at 0 °C, and stirring was continued for 1 h at room temperature, after which the reaction mixture was diluted with water and filtered. The filtrate was dried and concentrated to give the residue, chromatography of which on alumina with chloroform afforded **8a** (35 mg, 79%), colorless needles, mp 215—216 °C (EtOH) (lit.[8b] 217—218 °C). UV λ_{max}^{EtOH} nm (log ε): 230 (4.68), 280 (4.59), 312 (4.34). ^{1}H-NMR δ: 7.69, 7.50 (each 1H, AB-q, J=8.5 Hz, H-11 and H-12), 7.65, 7.31, 7.11, 6.79 (each 1H, each s, aromatic H), 6.04 (2H, s, OCH_2O), 4.14 (2H, s, H-6), 3.99, 3.95 (each 3H, each s, OMe×2), 2.60 (3H, s, NMe). MS m/z (%): 349 (M^+, 99), 348 (100), 332 (8.9), 318 (8.0), 304 (7.9), 291 (6.5), 174 (6.4). *Anal.* Calcd for $C_{21}H_{19}NO_4$: C, 72.19; H, 5.48; N, 4.01. Found: C, 72.16; H, 5.40; N, 4.03. The product was identical with authentic dihydronitidine on the basis of IR, MS and TLC comparisons.

2-Benzyloxy-5,6-dihydro-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridine (*O*-Benzyldihydrofagaronine) (8b)——LAH (200 mg, 5.2 mmol) was added portionwise to a stirred solution of **7b** (330 mg, 0.72 mmol) in dry THF (80 ml) in a stream of nitrogen, and the reaction mixture was stirred for 4 h at room temperature, after which it was diluted with water and filtered. The filtrate was concentrated and the residue was dissolved in methanol (50 ml). Sodium borohydride (200 mg, 5.2 mmol) was added to the methanol solution and the mixture was set aside for 1 h at room temperature. Methanol was evaporated off and the residue was taken up in methylene dichloride. The solution was washed with water and brine, dried, and concentrated to leave **8b** (303 mg, 95%), colorless needles, mp 205—206 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 231 (4.62), 278 (4.64), 312 (4.33). ^{1}H-NMR δ: 7.68 (1H, d, J=9 Hz, H-11), 7.58—7.20 (7H, m, aromatic H), 7.66, 7.16, 6.80 (each 1H, each s, aromatic H), 5.28 (2H, s, benzylic H), 4.15 (2H, s, H-6), 4.07, 3.98, 3.94 (each 3H, each s, OMe×3), 2.63 (3H, s, NMe). MS m/z (%): 441 (M^+, 100), 440 (17), 322 (28), 294 (20), 91 (17). *Anal.* Calcd for $C_{28}H_{27}NO_4$: C, 76.17; H, 6.16; N, 3.17. Found: C, 76.26; H, 6.06; N, 3.10.

8,9-Dimethoxy-5-methyl-2,3-methylenedioxybenzo[*c*]phenanthridinium Chloride (Nitidine Chloride) (1a)——A soluion of DDQ (125 mg, 0.55 mmol) in benzene (5 ml) was added to a vigorously stirred mixture of **8a** (94 mg, 0.27 mmol) and 5% aqueous sodium hydroxide (3 ml) in benzene (5 ml) at room temperature. Stirring was continued for 3 h at room temperature, the benzene layer was separated and the water layer was extracted with ethyl acetate. The combined organic layers were washed with water and brine, dried, and concentrated to dryness. Concentrated hydrochloric acid (5 ml) was added to the above residue, and the resulting precipitates were collected by filtration. The solids were recrystallized from methanol to afford **1a** (91 mg, 88%), yellow needles, mp 272—275 °C (MeOH) (lit.[8f] 274—278 °C). UV λ_{max}^{MeOH} nm (log ε): 222 (4.33), 236 (4.36), 272 (4.61), 292 (4.57), 301 (4.56), 330 (4.56), 386

(4.00). ^{1}H-NMR ($(CD_3)_2SO$) δ: 9.88 (1H, s, H-6), 8.90, 8.28 (each 1H, AB-q, J=8.5 Hz, H-11 and H-12), 8.36, 8.31, 7.90, 7.77 (each 1H, each s, aromatic H), 6.35 (2H, s, OCH_2O), 4.90 (3H, s, NMe), 4.24, 4.05 (each 3H, each s, OMe×2). MS m/z (%): 333 (M^+−Me, 100), 290 (30), 247 (25), 50 (28). *Anal.* Calcd for $C_{21}H_{18}ClNO_4 \cdot H_2O$: C, 62.76; H, 5.02; N, 3.49. Found: C, 62.56; H, 5.32; N, 3.20. The product was identical with natural nitidine chloride on the basis of mixed melting point determination, and ^{1}H-NMR, MS and TLC comparisons.

2-Benzyloxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridinium Chloride (*O*-Benzylfagaronine Chloride) (1b)——*O*-Benzyldihydrofagaronine (**8b**) (100 mg, 0.23 mmol) was oxidized with DDQ (80 mg, 0.35 mmol) as described for **1a** to give **1b** (100 mg, 93%) after treatment with concentrated hydrochloric acid, yellow needles, mp 242—244 °C (MeOH–AcOEt). UV λ_{max}^{MeOH} nm (log ε): 220 (4.44), 237 (4.43), 274 (4.72), 288 (4.64), 330 (4.55), 392 (4.00). ^{1}H-NMR ($(CD_3)_2SO$) δ: 9.93 (1H, s, H-6), 8.77 (1H, brs, H-11), 8.21 (2H, s, aromatic H), 8.11 (1H, s, aromatic H), 7.82 (2H, s, aromatic H), 7.69—7.32 (5H, m, aromatic H), 5.30 (2H, s, benzylic H), 4.99 (3H, s, NMe), 4.21, 4.09, 4.01 (each 3H, each s, OMe×3). MS m/z (%): 425 (M^+−Me, 40), 334 (100), 306 (32), 291 (19), 263 (23), 91 (45). *Anal.* Calcd for $C_{28}H_{26}ClNO_4 \cdot H_2O$: C, 68.08; H, 5.71; N, 2.84. Found: C, 67.93; H, 6.00; N, 2.54.

2-Hydroxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridinium Chloride (Fagaronine Chloride) (1c)——Hydrochloric acid (6 N, 6 ml) was added to a solution of **1b** (100 mg, 0.21 mmol) in ethanol (6 ml) and the reaction mixture was refluxed for 4 h. After evaporation of the solvent, the residual solid was recrystallized from methanol–ethyl acetate to afford **1c** (79 mg, 97%), yellow needles, mp 266—269 °C (186—190 °C)[19] [lit.[5a] 255 °C (202 °C)[19]; lit.[9a] 260—261 °C (198—200 °C)[19]; lit.[9b] 276 °C (193—195 °C)[19]]. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH). UV λ_{max}^{MeOH} nm (log ε): 221 (4.36), 237 (4.34), 274 (4.61), 300 (4.53), 310 (4.52), 330 (4.51), 390 (4.01). ^{1}H-NMR ($(CD_3)_2SO$) δ: 10.56 (1H, s, OH), 9.89 (1H, s, H-6), 8.76, 8.15 (each 1H, AB-q, J=9 Hz, H-11 and H-12), 8.28, 8.13, 7.86, 7.57 (each 1H, each s, aromatic H), 4.98 (3H, s, NMe), 4.21, 4.09, 4.03 (each 3H, each s, OMe×3). MS m/z (%): 335 (M^+−Me, 100), 334 (47), 306 (15), 292 (15), 277 (14), 248 (12), 50 (12). *Anal.* Calcd for $C_{21}H_{20}ClNO_4 \cdot H_2O$: C, 62.45; H, 5.49; N, 3.46. Found: C, 62.32; H, 5.84; N, 3.29. The product was identical with natural fagaronine chloride on the basis of IR, ^{1}H-NMR and TLC comparisons.

5,6-Dihydro-2-hydroxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridine (Dihydrofagaronine) (8c)——Sodium borohydride (15 mg, 0.4 mmol) was added to a stirred solution of **1c** (10 mg, 0.026 mmol) in methanol (3 ml). The reaction mixture was stirred for 1 h at room temperature and the methanol was evaporated off. Water was added to the residue, which was then extracted with methylene dichloride. The extract was washed with water and brine, dried, and concentrated to dryness. The residual solid was recrystallized from methanol to afford **8c** (7.5 mg, 82%), colorless crystals, mp 196—199 °C (lit.[9b] 196—200 °C). IR ν_{max}^{KBr} cm^{-1}: 3550 (OH). UV λ_{max}^{MeOH} nm (log ε): 230 (4.57), 276 (4.59), 313 (4.29). ^{1}H-NMR δ: 7.70, 7.50 (each 1H, AB-q, J=9 Hz, H-11 and H-12), 7.64, 7.31, 7.26, 6.80 (each 1H, each s, aromatic H), 5.29 (1H, s, OH), 4.15 (2H, s, H-6), 4.07, 3.99, 3.95 (each 3H, each s, OMe×3), 2.62 (3H, s, NMe). MS m/z (%): 351 (M^+, 14), 350 (31), 349 (27), 348 (33), 334 (47), 322 (87), 294 (78), 279 (45), 91 (100). High-resolution mass Calcd for $C_{21}H_{21}NO_4$: 351.1470. Found: 351.1470.

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Quinolizidines. XIX.[1] Synthesis of (−)-9-Demethyltubulosine

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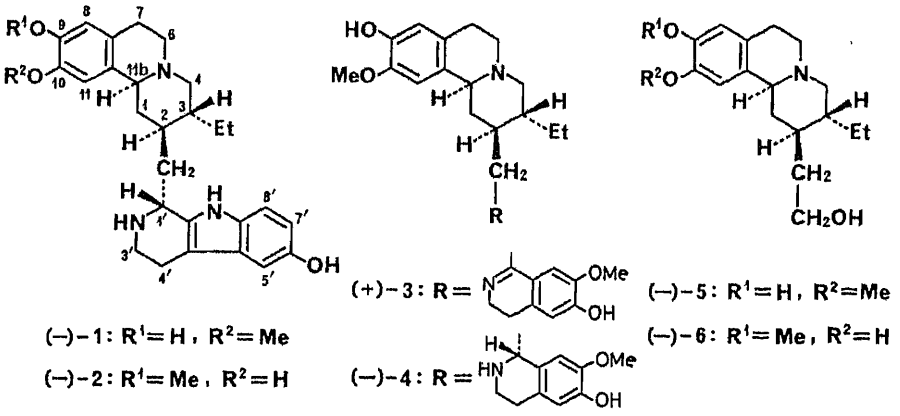
The chiral synthesis of (−)-9-demethyltubulosine [(−)-**1**] has been achieved for the first time *via* a "cincholoipon-incorporating route," which started from cincholoipon ethyl ester [(+)-**7**] and passed through the intermediates (−)-**8**, (−)-**9**, (+)-**10**, and (+)-**11**. As a result, the absolute configuration of the *Alangium vitiense* alkaloid 9-demethyltubulosine has been unequivocally established to be that represented by formula (−)-**1**.

Keywords——*Alangium vitiense* alkaloid; demethyltubulosine; chiral synthesis; demethylisotubulosine; diethyl phosphorocyanidate amide formation; Bischler-Napieralski cyclization; carbon–nitrogen double-bond catalytic reduction; benzyl ether hydrogenolysis; CD epimer differentiation

(−)-9-Demethyltubulosine [(−)-**1**][2] is an antitumor alkaloid isolated by Husson's group[3] from the trunk bark of *Alangium vitiense* (A. GRAY) BAILLON (Alangiaceae), a plant collected in Vanuatu, New Hebrides.[4] They have reported that this alkaloid increased the survival time of mice infected with leukemia L1210 or P388.[3b] Its chemical structure and relative stereochemistry were determined[4] by analysis of the spectral data and conclusively by a direct comparison with synthetic (±)-**1**.[5] As regards the absolute stereochemistry, that represented by formula (−)-**1** was assigned[4] on the basis of the similarity in circular dichroism (CD) spectrum to the known *A. lamarckii* alkaloid (−)-10-demethyltubulosine [(−)-**2**].[6] This paper details the results of our efforts toward a chiral synthesis of the candidate structure (−)-**1**, which have confirmed the correctness of the above stereochemical assignment.[7]

Our previous work has shown that unified chiral syntheses of all four groups of benzo[*a*]quinolizidine-type *A. lamarckii* alkaloids[8] are possible through routes adopting the "cincholoipon-incorporating strategy."[9] In view of the fact that the target structure (−)-**1**



falls within the 9-hydroxy-10-methoxybenzo[*a*]quinolizidine-type group, we tried to extend the scope of this synthetic strategy to include the synthesis of (−)-**1**. The starting point in the synthetic scheme was the known tricyclic amino acid (−)-**8**, a common key intermediate synthon utilized for our recent unified syntheses of 9-demethylpsychotrine [(+)-**3**],[10] 9-demethylcephaeline [(−)-**4**],[11] and 9-demethylprotoemetinol [(−)-**5**].[12] The tricycle (−)-**8** was available from cincholoipon ethyl ester [(+)-**7**][13] by the previously reported 11-step synthesis,[10] and the reaction sequence thereafter paralleled that employed by us[5] for the racemic synthesis of **1** from (±)-**8**.

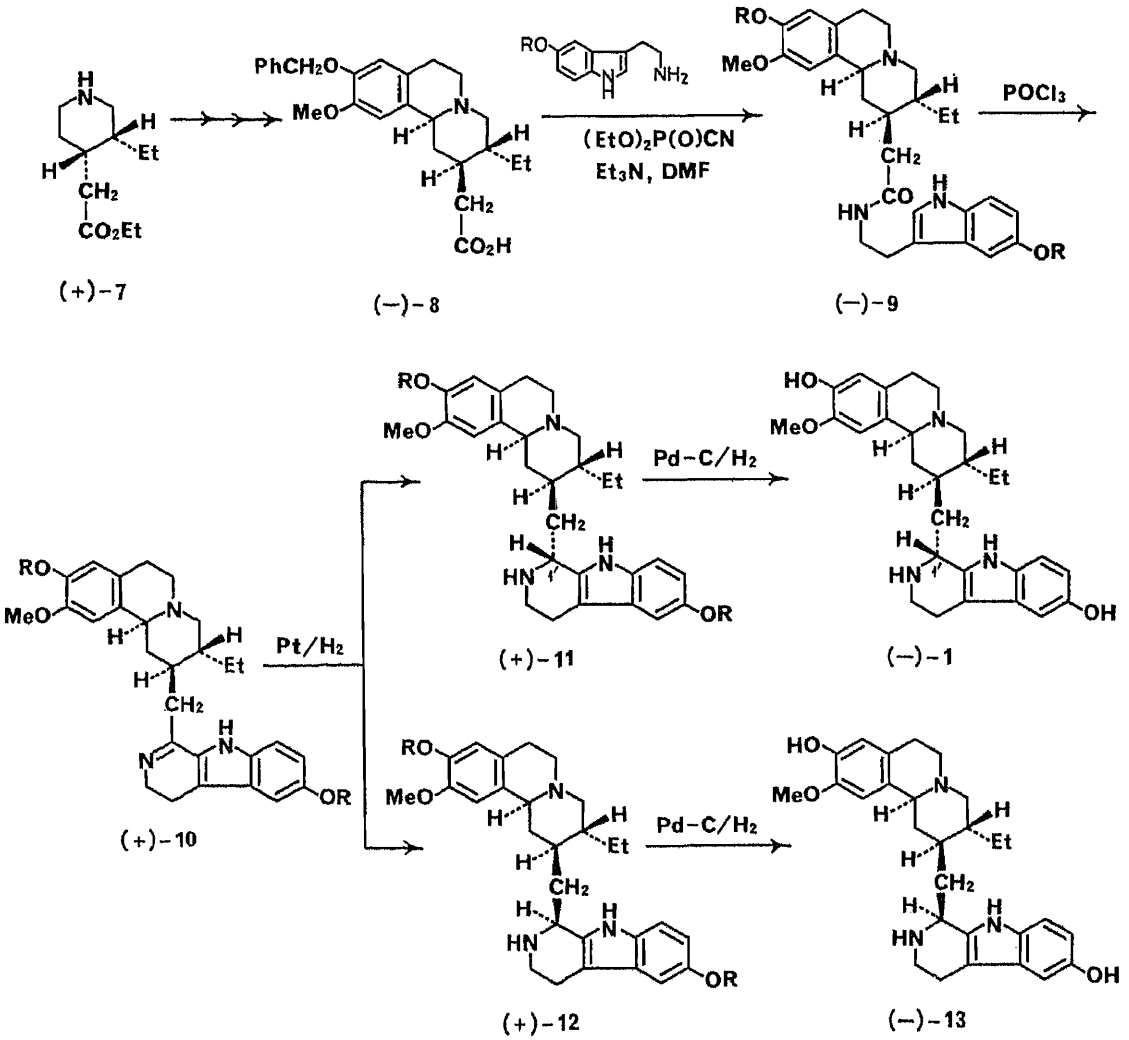


Chart 1

Thus, (−)-**8** and 5-benzyloxytryptamine[14] were coupled in *N*,*N*-dimethylformamide (DMF) by the diethyl phosphorocyanidate method,[15] giving the amide (−)-**9** in 86% yield. Bischler–Napieralski cyclization of (−)-**9** with $POCl_3$ in boiling toluene furnished the dihydro-β-carboline (+)-**10** (63% yield), which was then submitted to catalytic hydrogenation in dioxane over Adams catalyst. The resulting hydrogenation products were separated by column chromatography to give *O*,*O*-dibenzyl-9-demethyltubulosine [(+)-**11**] and its 1′-epimer [(+)-**12**] in 31% and 57% yields, respectively. On hydrogenolysis using hydrogen and

Pd–C catalyst, (+)-**11** produced the target molecule (−)-**1** in 90% yield. The epimeric base (+)-**12** was debenzylated similarly, providing 9-demethylisotubulosine [(−)-**13**] in 86% yield. The stereochemistry of the newly formed asymmetric center at C-1′ of (+)-**11**, (+)-**12**, (−)-**1**, and (−)-**13** was confirmed by the identity of their solution infrared (IR) (in $CHCl_3$)[16] and nuclear magnetic resonance (NMR) spectra and thin-layer chromatographic (TLC) mobility with those of the corresponding racemic modifications[5] of established stereochemistry. The synthetic (−)-**1** proved to be identical with a natural sample of the *A. vitiense* alkaloid 9-demethyltubulosine by a direct comparison of the TLC mobility and ultraviolet (UV), IR, and ^{1}H- and ^{13}C-NMR spectra, and especially of the specific rotation and CD spectrum.

In summary, the results of the above chiral synthesis have defined the absolute configurations of the four asymmetric centers in the $C_{28}H_{35}N_3O_3$ *A. vitiense* alkaloid as shown in formula (−)-**1**. Interestingly, the 10-demethyl isomer (−)-**2**[6] and 10-demethylprotoemetinol [(−)-**6**][12,17] as well as the 9-demethylated congeners, such as 9-demethylpsychotrine [(+)-**3**][6b,10] and 9-demethylprotoemetinol [(−)-**5**],[12,17,18] have been found in *Alangium lamarckii* THWAITES, another species of the same genus. It is hoped that the newly established synthetic route to (−)-**1** will facilitate the supply of a sufficient amount of this alkaloid for further assessment of its oncostatic activity and for other biological tests.

Experimental

General Notes——All melting points were taken on a Yamato MP-1 capillary melting point apparatus and are corrected. See ref. 1 for details of instrumentation and measurements. Microanalyses were performed by Mr. Y. Itatani and his associates at Kanazawa University. The following abbreviations are used: br=broad, d=doublet, dd=doublet-of-doublets, m=multiplet, s=singlet, sh=shoulder, t=triplet.

(2*R*,3*R*,11b*S*)-9-Benzyloxy-*N*-[2-(5-benzyloxy-1*H*-indol-3-yl)ethyl]-3-ethyl-1,3,4,6,7,11b-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine-2-acetamide [(−)-9]——A solution of (−)-**8**[10] (311 mg, 0.76 mmol) and 5-benzyloxytryptamine[14] (304 mg, 1.14 mmol) in $HCONMe_2$ (5 ml) was stirred under ice-cooling, and diethyl phosphorocyanidate[19] (248 mg, 1.52 mmol) and Et_3N (154 mg, 1.52 mmol) were added in that order. The mixture was stirred at room temperature for 6 h and extracted, after addition of H_2O (15 ml), with $CHCl_3$. The $CHCl_3$ extracts were washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to leave a brown glass. The glass was crystallized from AcOEt to give (−)-**9** (431 mg, 86%) as colorless needles. Recrystallization from AcOEt furnished an analytical sample, mp 168—168.5 °C; $[\alpha]_D^{19}$ −8.0° (*c*=0.50, EtOH); MS *m/z*: 657 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3490 and 3460 (NH's), 2810 and 2750 (*trans*-quinolizidine ring),[20] 1658 (amide CO); ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6.6 Hz, $CCH_2\underline{Me}$), 3.78 (3H, s, OMe), 5.09 (4H, s, two $OC\underline{H}_2Ph$'s), 5.54 (1H, t, *J*=5.5 Hz, CONH), 6.61 (1H, s, $H_{(8)}$ or $H_{(11)}$), 6.70 (1H, s, $H_{(11)}$ or $H_{(8)}$), 6.93 (1H, dd, *J*=9.3, 2.2 Hz, $H_{(6')}$), 6.97 (1H, d, *J*=2.4 Hz, $H_{(2')}$), 7.12 (1H, d, *J*=2.2 Hz, $H_{(4')}$), 7.22 (1H, d, *J*=9.3 Hz, $H_{(7')}$), 7.2—7.5 (10H, m, two $OCH_2\underline{Ph}$'s), 8.07 (1H, br, indole NH).[21] *Anal.* Calcd for $C_{42}H_{47}N_3O_4$: C, 76.68; H, 7.20; N, 6.39. Found: C, 76.62; H, 7.28; N, 6.39.

(2*R*,3*R*,11b*S*)-9-Benzyloxy-2-[(6-benzyloxy-4,9-dihydro-3*H*-pyrido[3,4-*b*]indol-1-yl)methyl]-3-ethyl-1,3,4,6,7,11b-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizine [(+)-10]——A solution of (−)-**9** (1.45 g, 2.2 mmol) and $POCl_3$ (3.37 g, 22 mmol) in dry toluene (60 ml) was heated under reflux in an atmosphere of nitrogen for 2.5 h. The reaction mixture was concentrated *in vacuo*, and 5% aqueous KOH (50 ml) and CH_2Cl_2 (50 ml) were added to the oily residue under ice-cooling. The CH_2Cl_2 layer was separated from the aqueous layer, which was further extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to leave a brown glass. Purification of the glass by column chromatography [alumina, CH_2Cl_2–EtOH (50:1, v/v)] afforded (+)-**10** (892 mg, 63%) as a pale yellow glass, $[\alpha]_D^{27}$ +34.6° (*c*=1.00, EtOH); MS *m/z*: 639 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3490 (NH), 2760 (*trans*-quinolizidine ring)[20]; ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, t, *J*=6.8 Hz, $CCH_2\underline{Me}$), 3.53 (3H, s, OMe), 5.04 and 5.10 (2H each, s, two $OC\underline{H}_2Ph$'s), 6.41 (1H, s, $H_{(8)}$ or $H_{(11)}$), 6.55 (1H, s, $H_{(11)}$ or $H_{(8)}$), 6.9—7.5 (13H, m, $H_{(5')}$, $H_{(7')}$, $H_{(8')}$, and two $OCH_2\underline{Ph}$'s), 8.34 (1H, br, NH).[22]

[2*S*-[2α(*S),3β,11bβ]]- and [2*S*-[2α(*R**),3β,11bβ]]-9-Benzyloxy-2-[(6-benzyloxy-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-1-yl)methyl]-3-ethyl-1,3,4,6,7,11b-hexahydro-10-methoxy-2*H*-benzo[*a*]quinolizines [(+)-11 and (+)-12]**——A solution of (+)-**10** (608 mg, 0.95 mmol) in dioxane (15 ml) was hydrogenated over Adams catalyst (100 mg) at atmospheric pressure and 29 °C for 1.5 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to leave an orange oil, which was dissolved in $CHCl_3$ (50 ml). The $CHCl_3$ solution was washed successively with 5% aqueous NaOH and saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The resulting pale orange glass was then chromatographed on a silica gel column using $CHCl_3$–EtOH (10:1, v/v) as the eluent. Earlier fractions gave *O,O*-dibenzyl-9-demethyltubulosine [(+)-**11**] (191 mg, 31%) as a pale

yellowish glass, $[\alpha]_D^{24}$ +7.6° (c = 1.00, EtOH); MS m/z: 641 (M^+). The IR ($CHCl_3$) and ^{1}H-NMR ($CDCl_3$) spectra of this sample were identical with those of authentic (±)-**11**.[5]

Later fractions in the above chromatography provided the 1′α-H isomer (+)-**12** (346 mg, 57%) as a pale yellowish glass, $[\alpha]_D^{24}$ +6.8° (c = 1.00, EtOH); MS m/z: 641 (M^+). The IR ($CHCl_3$) and ^{1}H-NMR ($CDCl_3$) spectra of this specimen were superimposable on those of authentic (±)-**12**.[5]

[2*S*-[2α(*S),3β,11bβ]]-3-Ethyl-1,3,4,6,7,11b-hexahydro-9-hydroxy-2-[(6-hydroxy-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-1-yl)methyl]-10-methoxy-2*H*-benzo[*a*]quinolizine [(−)-9-Demethyltubulosine] [(−)-1]**——A solution of (+)-**11** (205 mg, 0.32 mmol) in MeOH–AcOH (1 : 1, v/v) (15 ml) was hydrogenated over 10% Pd–C (200 mg) at atmospheric pressure and 24 °C for 3 h. Removal of the catalyst by filtration and evaporation of the filtrate under reduced pressure left a pale yellowish oil, which was triturated with H_2O (5 ml). The resulting aqueous mixture was filtered, and the filtrate was made alkaline with 10% aqueous Na_2CO_3. The precipitate that resulted was filtered off, washed with H_2O, and dried to give (−)-**1** (132 mg, 90%) as a pale yellowish solid. Purification of the solid by column chromatography [alumina, $CHCl_3$–MeOH (10 : 1, v/v)] and trituration of the resulting yellow glass with ether produced a slightly yellowish powder, mp 203—205 °C; $[\alpha]_D^{25}$ −81.0° (c = 1.00, pyridine); MS m/z: 461 (M^+); CD ($c = 8.23 \times 10^{-5}$ M, EtOH) $[\theta]^{22}$ (nm): 0 (318), +300 (314) (pos. max.), 0 (310), −2920 (295) (neg. max.), −2190 (288) (pos. max.), −3280 (277) (neg. max.), −120 (250) (pos. max.). The IR (Nujol), UV (MeOH, 0.1 N aqueous NaOH, 0.1 N aqueous HCl), ^{1}H-NMR (Me_2SO-d_6), and ^{13}C-NMR (Me_2SO-d_6) spectra and TLC mobility of this sample were identical with those (excepting the solid-state IR spectrum) of authentic (±)-**1**[5] as well as those of the $C_{28}H_{35}N_3O_3$ alkaloid [lit.[3,4] mp 200 °C; lit.[3,4] $[\alpha]_D^{20}$ −40° (c = 1, pyridine); CD ($c = 8.40 \times 10^{-5}$ M, EtOH) $[\theta]^{18}$ (nm): 0 (318), +240 (314) (pos. max.), 0 (310), −2740 (294) (neg. max.), −2080 (288) (pos. max.), −2800 (276) (neg. max.), 0 (250) (pos. max.)[23]] isolated from *Alangium vitiense*.[3,4]

[2*S*-[2α(*R),3β,11bβ]]-3-Ethyl-1,3,4,6,7,11b-hexahydro-9-hydroxy-2-[(6-hydroxy-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-1-yl)methyl]-10-methoxy-2*H*-benzo[*a*]quinolizine [(−)-9-Demethylisotubulosine] [(−)-13]**——Debenzylation of (+)-**12** and work-up of the reaction mixture were performed as described above for (−)-**1**, giving (−)-**13** (86% yield) as a slightly yellowish powder, mp 200—202 °C; $[\alpha]_D^{25}$ −98.8° (c = 1.00, pyridine); MS m/z: 461 (M^+); CD ($c = 5.51 \times 10^{-5}$ M, EtOH) $[\theta]^{22}$ (nm): 0 (325), −4720 (310) (neg. max.), −3630 (300) (sh), 0 (294), +8890 (278) (pos. max.), +1270 (248) (neg. max.), +3630 (242) (pos. max.), 0 (238). The UV (MeOH, 0.1 N aqueous NaOH, 0.1 N aqueous HCl) and ^{1}H-NMR (Me_2SO-d_6) spectra and TLC mobility of this sample were identical with those of authentic (±)-**13**.[5]

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Plant Mucilages. XL.[1] A Representative Mucilage, "Hibiscus-Mucilage SF," from the Flower Buds of *Hibiscus syriacus*

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A representative mucilage, named Hibiscus-mucilage SF, was isolated from the white flower buds of *Hibiscus syriacus* L. It was homogeneous on electrophoresis and gel chromatography. Its intrinsic viscosity value in aqueous solution was 26.0. It is mainly composed of partially acetylated acidic polysaccharide of molecular weight 1050000, and is composed of L-rhamnose : D-galactose : D-galacturonic acid : D-glucuronic acid in the molar ratio of 36 : 36 : 33 : 22. Methylation analysis of both the mucilage and the carboxyl-reduced derivative, and partial hydrolysis studies enabled elucidation of the structural features.

Keywords——Hibiscus-mucilage SF; bud; *Hibiscus syriacus*; intrinsic viscosity; acidic polysaccharide; component analysis; carboxyl reduction; methylation analysis; partial hydrolysis; structural feature

The white flower bud of *Hibiscus syriacus* L. is an Oriental crude drug (Japanese name, Mokukinka) used as a demulcent and antidiarrheic. As constituents of the bud of this plant, saponarin,[2] β-carotene and lutein,[3] and an essential oil containing 65 components[4] have been reported so far. In addition, it is well known that the bud contains relatively large amounts of mucilages, but no structural study on the mucilages has previously been reported. We have now isolated a representative mucilage from the white flower buds of this plant. Its properties and structural features are reported here.

The fresh buds were homogenized and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol, then dissolved again in water. The solution was treated with sodium lauryl sulfate and sodium chloride. After centrifugation, the supernatant obtained was poured into acetone. The precipitate obtained was dissolved in water and the solution was dialyzed and purified by gel chromatography with Sephadex G-25, then the eluate was lyophilized.

The mucilage gave a single spot on zone electrophoresis with glass-fiber paper, and it gave a single peak on gel chromatography with Sephacryl S-400. Further, it gave a clear band on polyacrylamide gel disk electrophoresis. Both periodate-Schiff reagent and the Coomassie blue reagent revealed the band in the same position. The mucilage had $[\alpha]_D^{24}+30.0°$ (0.1% NH_4OH, $c=0.1$), and its aqueous solution gave the high intrinsic viscosity value of 26.0 at 30°C. Gel chromatography with standard dextrans gave a value of about 1050000 for the molecular weight. The name "Hibiscus-mucilage SF" is proposed for this substance.

As component sugars of the mucilage, rhamnose, galactose, galacturonic acid, and glucuronic acid were identified. Quantitative determination showed that the mucilage contained 20.2% rhamnose, 22.2% galactose, 22.5% galacturonic acid, and 15.2% glucuronic acid. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a signal at δ 2.13, suggesting the presence of *O*-acetyl groups. This was confirmed by gas chromatography (GC) of the hydrolyzate, and the acetyl content was determined to be 5.9%. The molar ratio of rhamnose : galactose : galacturonic acid : glucuronic acid : *O*-acetyl is 36 : 36 : 33 : 22 : 36. De-

termination of protein content was carried out by the method of Lowry *et al.*,[5] and a value of 8.1% was obtained.

The carboxyl groups of hexuronic acids in the mucilage were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues.[6] Methylation of the original mucilage and the carboxyl-reduced derivative was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[7] The methylated products were hydrolyzed, and the hydrolyzates were converted into the partially methylated alditol acetates.[8] Methyl ethers of hexuronic acids were removed from the hydrolyzate of the methylated original mucilage by treatment with an anion-exchange resin. Gas-liquid chromatography (GLC)-mass spectrometry (MS)[9] revealed derivatives of 3,4-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,6-tri-*O*-methyl-D-galactose as the products in a molar ratio of 1 : 1 : 1 : 1 from the original mucilage. Alditol acetates of 3,4-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-L-rhamnose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose, and 2,6-di-*O*-methyl-D-galactose were identified in a molar ratio of 18 : 18 : 22 : 18 : 29 : 22 from the carboxyl-reduced product.

These results suggested that the minimal repeating unit of the polysaccharide moiety of the mucilage is composed of seven kinds of component sugar units as shown in Chart 1.

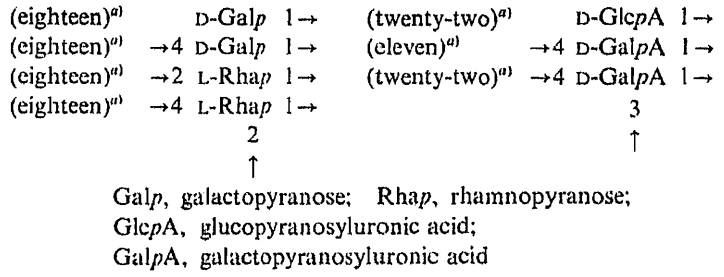

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Hibiscus-Mucilage SF

a) Number of residues.

The mucilage was partially hydrolyzed with dilute sulfuric acid, and then neutralized and treated with Dowex 50W (H^+). The eluate with water was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (formate form). In addition to a part of the component monosaccharides, three oligosaccharides (I to III) were obtained by stepwise elution with dilute formic acid. Based on the results of component sugar analysis and a comparison of their chromatographic properties, their ^{1}H-NMR spectra, and their values of specific rotation with those of authentic samples,[10] I to III were identified as the following three oligosaccharides (Chart 2).

All galactose residues were liberated from the mucilage under the conditions of partial hydrolysis described above. In conjunction with the results of methylation analysis, this finding suggests that a half of the rhamnose residues in the backbone chain possesses a 1→4

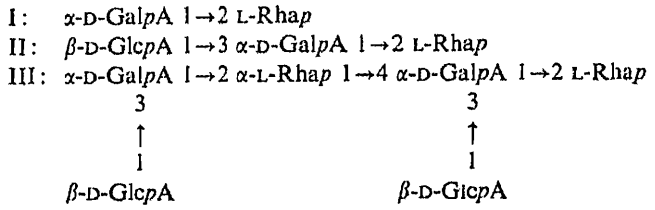

Chart 2. Structural Features of Oligosaccharides I—III

galactosyl galactose chain at position 4. A part (5.3%) of the rhamnose residues was also liberated by partial hydrolysis. The value of specific rotation of the galactose fraction was consistent with the D configuration.

The ^{1}H-NMR spectrum of the mucilage showed four anomeric proton signals at δ 4.62 (d, J=7 Hz), δ 4.71 (d, J=7 Hz), δ 4.98 (d, J=2 Hz), and δ 5.18 (br s). Their integral ratio was 3:2:3:3. The signals at δ 4.71, 4.98, and 5.18 were assigned to the anomeric protons of β-D-glucuronic acid, α-L-rhamnose, and α-D-galacturonic acid, respectively.[10] Thus, it can be concluded that the anomeric signal at δ 4.62 is due to β-D-galactose residues.

Based on the accumulated evidence described here, it may be concluded that the polysaccharide moiety of the mucilage contains the units shown in Chart 3.

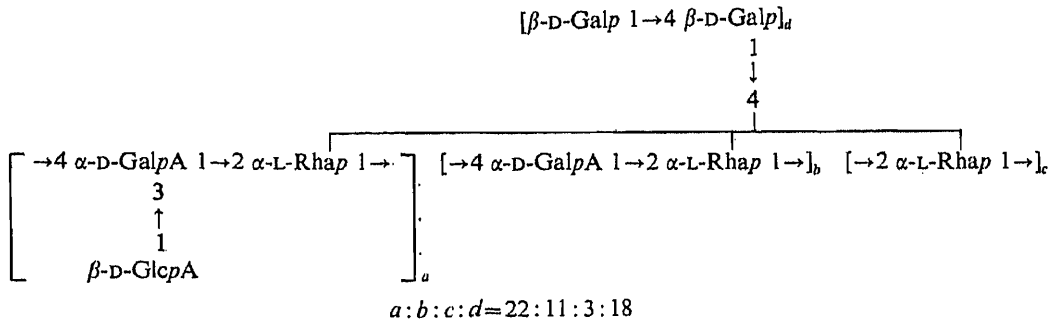

Chart 3. A Possible Structural Fragment of the Polysaccharide Moiety of Hibiscus-Mucilage SF

The component unit having the repeating structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose is common and major in all mucilages[1,10-17] except Okra-mucilage F[18] obtained by us from plants in the Malvaceae family. Okra-mucilage F from the immature fruits of *Abelmoschus esculentus* has a backbone chain consisting of the (1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose unit. This unit was also found in the backbone chains of Althaea-mucilage OL[11] from the leaves of *Althaea officinalis*, Abelmoschus-mucilage G[12] from the roots of *Abelmoschus glutinotextilis*, and Hibiscus-mucilage SL[1] from the leaves of *Hibiscus syriacus*, in addition to Okra-mucilage F. α-1→4-Linked L-rhamnopyranosyl L-rhamnopyranose residues were found in the backbone chains of Althaea-mucilage OL,[11] and Althaea-mucilages R[13] and RL[14] from the roots and the leaves of *Althaea rosea*, Abelmoschus-mucilage M[15] from the roots of *Abelmoschus manihot*, and Okra-mucilage R[16] from the roots of *Abelmoschus esculentus*. In the former studies on the mucilages from the plant sources in the Malvaceae family, Althaea-mucilage OL[11] was the sole example having the three types of units in the backbone chain. Thus Hibiscus-mucilage SF is the second example having the three units shown in Chart 3 in its main chain.

The presence of β-1→4-linked D-galactopyranosyl D-galactopyranose side chains at position 4 of a part of the rhamnose residues in the main chain is common in Hibiscus-mucilage SF, Althaea-mucilage O,[10] and Okra-mucilage F.[18]

We have obtained a hexasaccharide, a nonasaccharide, and a dodecasaccharide having the repeating structure of the trisaccharide unit, (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose, in addition to the trisaccharide and the disaccharide, α-D-galactopyranosyluronic acid-(1→2)-L-rhamnopyranose, by partial hydrolysis of Althaea-mucilage OL and most of the other mucilages from plants in the Malvaceae family. However, in the case of Hibiscus-mucilage SF, neither the nonasaccharide nor the dodecasaccharide was found in the partial hydrolyzate. Thus, the trisaccharide unit must be present in a dispersed condition as its

monomer or dimer in the backbone chain of Hibiscus-mucilage SF, and such a state in the main chain appears to be characteristic of this substance.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter. NMR spectra were recorded on a JEOL JNM-GX 270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70 °C. GC and GLC were carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-MS was performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrometer. Viscosity was determined with an Ubbelohde-type viscosimeter.

Material——The material was obtained at the end of August 1985 from a plant cultivated in Saitama prefecture. The fresh buds contained 86.3% water.

Isolation of the Mucilage——The fresh buds (50 g) were homogenized and extracted with water (500 ml) under stirring for 1 h at room temperature. After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was lyophilized; the yield of the crude mucilage was 1.0%. The crude mucilage was dissolved in water (500 ml) and 5% sodium lauryl sulfate (50 ml) was added, followed by sodium chloride (2.92 g) at 5 °C. After centrifugation, the supernatant was poured into two volumes of acetone. The precipitate was dissolved in water, then dialyzed against distilled water and concentrated. The solution was applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 54 were combined, concentrated and lyophilized. Yield, 60 mg. Hibiscus-mucilage SF was obtained as a white powder.

Glass-Fiber Paper Electrophoresis——This was performed as described in a previous report[19] on Whatman GF 83 glass-fiber paper at 570 V with the following two buffers: A, 0.08 M pyridine–0.04 M acetic acid (pH 5.4); B, 0.025 M borax: 0.1 N sodium hydroxide (10 : 1, pH 9.3). The sample gave a single spot at distances of 2.4 cm in buffer A (1.5 h) from the origin toward the anode and 3.5 cm in buffer B (45 min) from the origin toward the cathode.

Polyacrylamide Gel Electrophoresis——This was performed in an apparatus with gel tubes (4 × 145 mm each) and 0.005 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 1 h. Gels were stained for carbohydrate by the periodic acid-Schiff (PAS) procedure, and stained for protein with Coomassie blue. The sample gave a clear band with both reagents at a distance of 6.8 cm from the origin.

Gel Chromatography——The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 94 cm) of Sephacryl S-400. Elution was carried out with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method.[20] Standard dextrans having known molecular weights were run on the column to obtain a calibration curve.

Qualitative Analysis of Components——Hydrolysis, isolation and cellulose thin-layer chromatography (TLC) of component sugars were carried out as described in a previous report.[21]

Determination of Components——Neutral sugars in the original and the carboxyl-reduced mucilages were analyzed by GLC after hydrolysis and conversion into alditol acetates as described in a previous report.[15] Rhamnose was also determined by the thioglycolic acid method,[22] and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.[23]

Determination of *O*-Acetyl Groups——The sample was hydrolyzed with 0.2 N hydrochloric acid and subjected to GLC with a column (3 mm × 2 m long spiral glass) packed with 5% Thermon-1000–0.5% phosphoric acid on Chromosorb W at 120 °C as described in a previous report.[24]

Reduction of Carboxyl Groups——The mucilage (70 mg) was dissolved in water (50 ml), then 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (0.7 g) was added. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 N hydrochloric acid under stirring for 2 h, then 2 M sodium borohydride (7 ml) was added gradually to the reaction mixture during 4 h while the pH was maintained at 7.0 by titration with 4 N hydrochloric acid under stirring at room temperature. The solution was dialyzed against distilled water, then the non-dialyzable fraction was concentrated to 50 ml. The product was reduced five times more under the same conditions. The final non-dialyzable fraction was concentrated and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 27 to 44 were combined and lyophilized. Yield, 15.8 mg.

Methylation——This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[1] The methylation was repeated four times under the same conditions. Yields were 15.5 mg from 12 mg of the carboxyl-reduced product and 3 mg from 10 mg of the original mucilage.

Analysis of the Methylated Products——The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[18] GLC and GLC-MS of partially methylated alditol acetates were performed with a column (3 mm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 200 °C with a helium flow of 60 ml per min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and their main fragments in the

Table I. Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a]	Main fragments (*m/z*)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.89	43, 89, 129, 131, 189
1,2,4,5-Ac-3-Me-L-Rhamnitol	1.58	43, 87, 101, 129, 143, 189, 203
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,5-Ac-2,3,4,6-Me-D-Galactitol	1.14	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Galactitol	1.95	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.72	43, 45, 87, 117, 129

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac=acetyl; Me=methyl (*e.g.*, 1,2,5-Ac-3,4-Me- = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-).

Table II. Specific Rotations, Sugar Compositions, and *Rf* Values of Oligosaccharides

Oligosaccharide	$[\alpha]_D^{24}$ in H_2O	Sugar composition	TLC (*Rf*)
I	+93.2°	GalA : Rha = 1 : 1	0.44
II	+84.4°	GlcA : GalA : Rha = 1 : 1 : 1	0.36
III	+81.0°	GlcA : GalA : Rha = 1 : 1 : 1	0.26

Table III. Amino Acid Composition of Hibiscus-Mucilage SF (Molar Percent)

Aspartic acid	7.39	Methionine	2.56
Threonine	6.67	Isoleucine	6.41
Serine	5.98	Leucine	9.44
Glutamic acid	9.10	Tyrosine	3.08
Proline	5.21	Phenylalanine	4.10
Glycine	9.40	Lysine	5.34
Alanine	12.09	Histidine	1.63
Valine	7.48	Arginine	4.10

mass spectra are listed in Table I.

Partial Hydrolysis and Isolation of Oligosaccharides——The mucilage (100 mg) was suspended in 1 N sulfuric acid (20 ml) and heated in a boiling water bath for 2 h. After neutralization with barium carbonate, followed by filtration, the filtrate was passed through a column (1 × 5 cm) of Dowex 50WX8 (H^+). The eluate with water was concentrated and lyophilized (yield, 40 mg), then an aqueous solution of the lyophilizate was applied to a column (1 × 8 cm) of DEAE-Sephadex A25 (formate form). The column was eluted successively with water (25 ml), 0.1 M formic acid (75 ml), 0.2 M formic acid (65 ml), and 0.3 M formic acid (45 ml). Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. The eluates obtained from the column were divided into four groups: Frac. 1, tubes 1 to 3; frac. 2, tubes 10 to 17; frac. 3, tubes 21 to 33; frac. 4, tubes 34 to 42. The yields were 22.8 mg for frac. 1, 13.9 mg for frac. 2, 1.0 mg for frac. 3, and 0.3 mg for frac. 4. Frac. 1 contained galactose and rhamnose in a ratio of 19 : 1. Frac. 2 was dissolved in water and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water and fractions of 5 ml were collected. The eluates obtained from the column were divided into two groups: Frac. a, tubes 125 to 138; frac. b, tubes 141 to 160. The yields were 3.4 mg for frac. a and 6.3 mg for frac. b. Oligosaccharides I and II were obtained from fracs. b and a, respectively. Oligosaccharide III was obtained from fracs. 3 and 4 after purification on a column of Sephadex G-25 as described in a previous report.[13] Yield, 1.0 mg.

Analysis of the Oligosaccharides——Analysis of component sugars was performed as described in a previous report.[15] TLC was carried out on Merck precoated Kieselgel 60 plates using *n*-butanol–acetic acid–water (2 : 1 : 1, v/v) as a developing solvent as described in a previous report.[1] The results are listed in Table II.

Amino Acid Composition——Amino acids were determined by the method of Bidlingmeyer *et al.*[25] after hydrolysis with 6 N hydrochloric acid, and the composition found is given in Table III.

Acknowledgement We are grateful to Prof. M. Tomita, School of Pharmaceutical Sciences, Showa University, for the determination of amino acids. Financial support from the Ministry of Education, Science and Culture of Japan in the form of a Grant-in-Aid for Scientific Research is also gratefully acknowledged.

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Studies on Antitumor Agents. VII.[1] Antitumor Activities of *O*-Alkoxyalkyl Derivatives of 2′-Deoxy-5-trifluoromethyluridine

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Various *O*-alkoxyalkyl derivatives of 2′-deoxy-5-trifluoromethyluridine (F_3Thd) were synthesized, and the antitumor activities of the compounds against sarcoma 180 were examined by oral administration to mice. Among the formal-type derivatives, 3′,5′-di-*O*-ethoxymethyl (**3**), 3′,5′-di-*O*-benzyloxymethyl (**12**), 5′-*O*-benzyloxymethyl (**13**) and 3′-*O*-benzyloxymethyl (**14**) compounds showed high activities, which were six-fold higher than that of F_3Thd itself. Since acetal-type derivatives were unstable under acidic conditions, antitumor testing of the compounds was also carried out with co-administration of sodium bicarbonate. 5′-*O*-(1-Ethoxypropyl)-F_3Thd (**25**) and 5′-*O*-(1-benzyloxypropyl)-F_3Thd (**37**) showed the highest activities among the acetal-type derivatives, but the ED_{50} values of the compounds were not lower than those of effective formal-type compounds.

These *O*-alkoxyalkyl derivatives of F_3Thd are resistant to degradation by thymidine phosphorylase and are activated by microsomal drug-metabolizing enzymes after absorption.

Keywords——2′-deoxy-5-trifluoromethyluridine; protecting group; benzyloxymethylation; antitumor activity; drug-metabolizing enzyme

2′-Deoxy-5-trifluoromethyluridine (F_3Thd) was first synthesized by Heidelberger and his coworkers in 1962.[2] A metabolite of F_3Thd, 2′-deoxy-5-trifluoromethyluridine 5′-monophosphate (F_3TMP) has been reported to inhibit the enzyme (thymidylate synthetase).[3] It has also been reported that deoxyribonucleic acid (DNA) chain elongation and joining were inhibited by incorporation of another metabolite of F_3Thd, 2′-deoxy-5-trifluoromethyluridine 5′-triphosphate, into DNA.[4] As a result of these actions of the metabolites of F_3Thd, F_3Thd is a potent antiviral and antitumor agent in some systems.[5~7] However, F_3Thd is unsatisfactory for practical medicinal use in cancer chemotherapy, because of its short half-life in plasma.[8] Rapid metabolic degradation by thymidine phosphorylase has been reported to be responsible for this.[8,9] Therefore, depot forms of F_3Thd which resist degradation by the enzyme would be expected to maintain higher concentrations of F_3Thd in plasma and to show improved antitumor activity *in vivo*.

Recently, we have reported syntheses and antitumor activities of acyl derivatives of F_3Thd.[1] Acylation was effective in elevating the antitumor activity of F_3Thd, but the derivatives were rather easily saponified to F_3Thd by intestinal homogenate.

It has been reported that tetrahydrofuryl groups of 3′,5′-di-*O*-(tetrahydro-2-furyl)-thymidine can be removed by microsomal drug-metabolizing enzymes.[10] Alkoxyalkyl substituents on 5-fluorouracil have also been reported to be removed by the enzyme in the same way as the tetrahydrofuryl group of 1-(tetrahydro-2-furyl)-5-fluorouracil (tegafur, which is widely used clinically).[11] These observations suggest that *O*-alkoxyalkyl derivatives

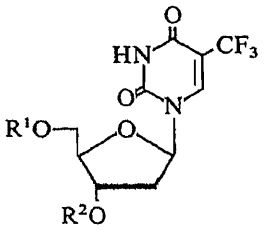
of F_3Thd would resist degradation by thymidine phosphorylase and be activated slowly after absorption, possibly giving a high therapeutic index.

Therefore, various *O*-alkoxyalkyl derivatives of F_3Thd were synthesized and their antitumor activities on oral administration were evaluated to find candidate compounds for clinical use, especially for maintenance therapy after surgical treatment like other depot forms of 5-fluorouracil. This paper describes the synthesis of such derivatives of F_3Thd and the antitumor activities of the compounds against sarcoma 180.

Results and Discussion

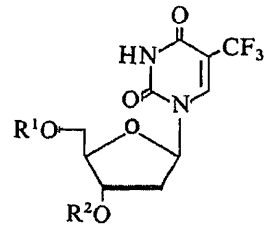
The structures and antitumor activities of *O*-(tetrahydro-2-furyl) (**1**) and *O*-(tetrahydro-2-pyranyl) (**2**) compounds and formal-type derivatives of F_3Thd are shown in Table I. Compound **1** was obtained by the reaction of F_3Thd and 2,3-dihydrofuran, with *p*-toluenesulfonic acid (TsOH) as a catalyst, in dioxane. Treatment of F_3Thd with an excess of 2-methoxytetrahydropyran and a small amount of TsOH in dioxane under heating gave a mixture of 5′-*O*- (**2**), 3′-*O*- and 3′,5′-di-*O*-(tetrahydro-2-pyranyl)-F_3Thd. The products were purified by silica gel column chromatography. Compounds **3**—**14** were similarly synthesized by the reaction of F_3Thd and dialkoxymethane using TsOH. The dialkoxymethanes were obtained by treatment of methylene chloride with an excess of sodium hydroxide and alcohol

TABLE I. Antitumor Effects of Formal-Type Compounds of F_3Thd



Compd.	R^1	R^2	ED_{50} (mg/kg/d)
1		R^1	42
2		H	46
3	$CH_2OC_2H_5$	R^1	12
4	$CH_2OC_2H_5$	H	23
5	CH_2OCH_3	H	36
6	CH_2SCH_3	H	>40
7	$CH_2O(CH_2)_3CH_3$	H	23
8	$CH_2OCH_2CH_2SCH_3$	H	>80
9	H	$CH_2OCH_2CH_2SCH_3$	>80
10	CH_2OCH_2- 	H	46
11	H	CH_2OCH_2- 	>80
12	CH_2OCH_2- 	R^1	12
13	CH_2OCH_2- 	H	10
14	H	CH_2OCH_2- 	11
F_3Thd			63

TABLE II. Antitumor Effects of Acetal-Type Compounds of F_3Thd



Compd.	R^1	R^2	ED_{50} (mg/kg/d)
15	$CH(CH_3)CH_2CH(CH_3)_2$	H	>40
16	$CH(CH_3)O$-(H)	H	>40
17	$CH(CH_3)OCH_2$-(H)	R^1	>40
18	$CH(CH_3)OCH_2$-(H)	H	>40
19	H	$CH(CH_3)OCH_2$-(H)	>40
20	$CH(CH_3)O(CH_2)_5CH_3$	H	34
21	H	$CH(CH_3)O(CH_2)_5CH_3$	>40
22	$CH(CH_3)O(CH_2)_9CH_3$	H	>40
23	$CH(CH_3)OCH_2$-(O)	H	>40
F_3Thd			63

at 140 °C in a sealed stainless steel tube.

Acetals were synthesized by the reaction of aldehyde and alcohol in the presence of calcium chloride at room temperature. Treatment of F_3Thd in dioxane with acetals under the same reaction conditions as used for alkoxymethylation gave compounds **15**—**43**.

The structures and antitumor activities of those componds are shown in Tables II, III and V.

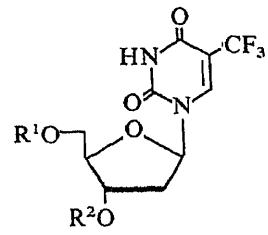
Biological Activity

Compounds **1** and **2** were slightly more effective than F_3Thd, and some of the formal-type derivatives showed high activities. The ED_{50} values of the 3′,5′-di-*O*-ethoxymethyl (**3**), 3′,5′-di-*O*-benzyloxymethyl (**12**), 5′-*O*-benzyloxymethyl (**13**) and 3′-*O*-benzyloxymethyl (**14**) compounds were 12, 12, 10 and 11 mg/kg/d, respectively. These results show that the antitumor activity of F_3Thd can be elevated by *O*-ethoxymethylation or *O*-benzyloxymethylation to 6 times that of F_3Thd itself.

Compounds **6**, **8** and **9** with chains containing a methylthio group showed no activity at the same dose as that of F_3Thd. These compounds are presumably not activated by microsomal drug-metabolizing enzymes.

Three metabolic pathways can be assumed for the activation of formal-type derivatives of F_3Thd (Chart 1). One is the oxidative route which results in release of benzaldehyde and formaldehyde. Oxidation of another carbon would release benzyl alcohol and formyl ester,

TABLE III. Antitumor Effects of Acetal-Type Compounds of F_3Thd

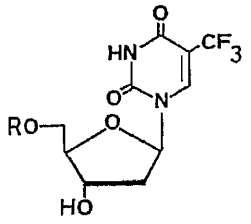


Compd.	R^1	R^2	ED_{50} (mg/kg/d)
24	$CH(CH_3)OC_2H_5$	H	51
25	$CH(CH_2CH_3)OC_2H_5$	H	29
26	$CH(C_6H_5)OC_2H_5$	H	—
27	$CH(CH_2C_6H_5)OC_2H_5$	H	>40
28	$CH(CH_2CH_2C_6H_5)OC_2H_5$	H	>37
29	$CH(C(CH_3)_3)OC_2H_5$	H	40
30	$CH(CH_3)OCH_2C_6H_5$	R^1	26
31	$CH(CH_3)OCH_2C_6H_5$	H	25
32	H	$CH(CH_3)OCH_2C_6H_5$	31
33	$CH(CH_3)OCH_2C_6H_4$-C_4H_9	R^1	>40
34	$CH(CH_3)OCH_2C_6H_4$-C_4H_9	H	27
35	H	$CH(CH_3)OCH_2C_6H_4$-C_4H_9	>40
36	$CH_2OCH_2CH_2C_6H_5$	H	32
37	$CH(CH_2CH_3)OCH_2C_6H_5$	H	22
38	$CH(CH(CH_3)_2)OCH_2C_6H_5$	H	36
39	H	$CH(CH(CH_3)_2)OCH_2C_6H_5$	>20
40	$CH(C(CH_3)_3)OCH_2C_6H_5$	H	>40
41	H	$CH(C(CH_3)_3)OCH_2C_6H_5$	>40
F_3Thd			63

TABLE IV. Antitumor Effects of F_3Thd Derivatives with Co-administration of Sodium Bicarbonate

Compd.	ED_{50} (mg/kg/d) Alone	$NaHCO_3$ added	Compd.	ED_{50} (mg/kg/d) Alone	$NaHCO_3$ added
4	23	19	**28**	37	31
13	12	12	**29**	>40	20
18	>40	>20	**31**	25	17
20	34	>20	**34**	27	>20
22	>40	>20	**37**	22	30
23	>40	>20	**38**	36	35
24	51	40	**40**	>40	24
25	29	19	**43**	>40	>20
27	>40	38			

TABLE V. Antitumor Effects of 1-Phenethyloxy Compounds of F_3Thd



Compd.	R	ED_{50} (mg/kg/d) Alone	$NaHCO_3$ added
13	CH_2OCH_2-Ph	10	12
31	$CH(CH_3)OCH_2$-Ph	25	17
42	$CH_2OCH(CH_3)$-Ph	25	—
43	$CH(CH_3)OCH(CH_3)$-Ph	40	20
36	$CH_2OCH_2CH_2$-Ph	32	—

which would eventually give F_3Thd. The third route would be direct hydrolysis, giving benzyl alcohol, formaldehyde and F_3Thd. The role of these metabolites in the toxicity of F_3Thd derivatives is not clear. Therefore, various acetal-type derivatives of F_3Thd were synthesized with the aim of obtaining more effective compounds with ED_{50} values of 10 mg/kg/d or less.

Since there is expected to be a parallel relation between antitumor activity and F_3Thd-releasing capability by the drug-metabolizing enzymes, F_3Thd derivatives which have a lipophilic activated site were synthesized and tested on S 180 (Table II). The ED_{50} values of most of the compounds were equal to that of F_3Thd or higher.

Various ethoxyalkyl and benzyloxyalkyl derivatives were next synthesized, in view of the high activities of the ethyl- and benzyl-formal-type compounds (Table III). Among the ethoxyalkyl-type compounds (**24**—**29**), *O*-(1-ethoxypropyl)-F_3Thd (**25**) showed the highest activity. Among the benzyloxy-type compounds (**30**—**41**), *O*-(1-benzyloxypropyl)-F_3Thd (**37**) was expected to show the highest activity because of the similarity of the structure, and this was indeed the case. Nevertheless, the ED_{50} values of both compounds (**25** and **37**) were

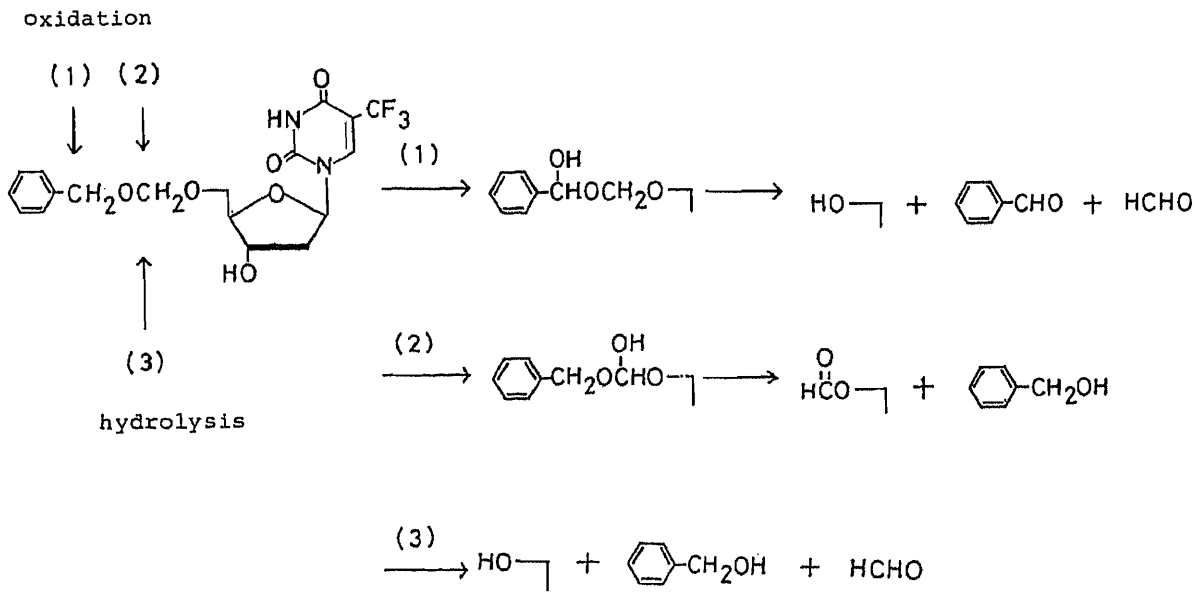


Chart 1

rather high (29 and 22 mg/kg/d, respectively) compared with those of the effective formal-type derivatives.

Since these alkoxyalkyl derivatives of F_3Thd are generally unstable under acidic conditions, some of the compounds would be hydrolyzed to F_3Thd in the gastro-intestinal tract before absorption, resulting in rather lower antitumor activities. Therefore, various F_3Thd derivatives were next examined with co-administration of sodium bicarbonate to prevent acidic hydrolysis. The results are summarized in Table IV.

Compounds **4** and **13** were not affected by co-administration of sodium bicarbonate, because those compounds were reasonably stable under acidic conditions. The antitumor activities of compounds **18**, **20**, **22** and **23** were also not elevated by sodium bicarbonate. These compounds may not be activated well by the microsomal drug-metabolizing enzymes. In contrast, co-administration of sodium bicarbonate decreased the ED_{50} values of compounds **24**, **25**, **27**, **28** and **29** by about 10 mg/kg/d. The antitumor activities of compounds **31** and **40**, among the compounds having a benzyloxy group, were also elevated by the co-administration. However, among the acetal-type compounds, none showing ED_{50} values in the region of 10 mg/kg/d could be obtained.

5′-*O*-(1-Phenethyloxy)methyl-F_3Thd (**42**) and 5′-*O*-1-(1-phenethyloxy)ethyl-F_3Thd (**43**) were next synthesized and compared with compounds **13**, **31** and **36** to elucidate the oxidation site at the first step of the metabolic pathway. As shown in Table V, the introduction of a methyl group into either formalic methylene or benzylic methylene decreased the antitumor activities. The ED_{50} value of compound **43** with two methyl groups was the highest among the compounds.

These results suggest that oxidative activation of the F_3Thd derivatives by the enzyme occurs at both the formalic and benzylic positions, and is retarded by the introduction of a methyl group.

Experimental

Chemicals——Melting points were determined with a Yanagimoto MP-3 micro melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a JEOL LMN-FX 100 spectrometer (using tetramethylsilane as an internal standard). Formals and acetals were purchased or synthesized by

TABLE VI. Physical Constants and Analytical Data of Alkoxyalkyl Derivatives of F_3Thd

Compd.	mp (°C)	Recryst. solvent	Yield (%)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
1	155—159	EtOH	67	$C_{18}H_{23}F_3N_2O_7$	49.54	5.31	6.42
					(49.57	5.47	6.46)
2	Foam		18	$C_{15}H_{19}F_3N_2O_6$	47.37	5.04	7.37
					(47.18	5.12	7.27)
3	Oil		19	$C_{16}H_{23}F_3N_2O_7$	46.60	5.62	6.79
					(46.64	5.69	6.91)
4	185—187	$CHCl_3$	17	$C_{13}H_{17}F_3N_2O_6$	44.07	4.84	7.91
					(44.12	4.92	8.00)
5	179—182	EtOH	19	$C_{12}H_{15}F_3N_2O_6$	42.36	4.44	8.23
					(42.39	4.51	8.35)
6	180.5—182.5	EtOH	15	$C_{12}H_{15}F_3N_2O_5S$	40.45	4.24	7.86
					(40.17	4.20	7.73)
7	187.5—188.5	EtOH	19	$C_{15}H_{21}F_3N_2O_6$	47.12	5.54	7.33
					(46.89	5.52	7.26)
8	160.5—161.5	EtOH	13	$C_{14}H_{19}F_3N_2O_6S$	42.00	4.78	7.00
					(41.72	4.76	6.96)
9	Oil		9	$C_{14}H_{19}F_3N_2O_6S$	42.00	4.78	7.00
					(41.78	4.68	6.88)
10	155—156	$CHCl_3$–EtOH	18	$C_{16}H_{21}F_3N_2O_7$	46.83	5.16	6.83
					(46.69	5.11	6.69)
11	Oil		14	$C_{16}H_{21}F_3N_2O_7$	46.83	5.16	6.83
					(46.66	5.10	6.65)
12	Oil		15	$C_{26}H_{27}F_3N_2O_7$	58.21	5.07	5.22
					(57.98	5.02	5.11)
13	184.5—186	EtOH	24	$C_{18}H_{19}F_3N_2O_6$	51.93	4.60	6.73
					(51.84	4.54	6.71)
14	Oil		23	$C_{18}H_{19}F_3N_2O_6$	51.93	4.60	6.73
					(51.88	4.61	6.69)
15	Foam		20	$C_{15}H_{19}F_3N_2O_6$	47.37	5.03	7.37
					(47.20	4.96	7.27)
16	Foam		23	$C_{18}H_{25}F_3N_2O_6$	51.18	5.97	6.42
					(51.00	5.88	6.22)
17	Oil		16	$C_{28}H_{43}F_3N_2O_7$	58.32	6.24	6.42
					(58.00	6.08	6.36)
18	163—165	EtOH	22	$C_{19}H_{27}F_3N_2O_6$	52.29	6.24	6.42
					(52.33	6.11	6.40)
19	Oil		12	$C_{19}H_{27}F_3N_2O_6$	52.29	6.24	6.42
					(52.22	6.13	6.40)
20	Foam		34	$C_{18}H_{27}F_3N_2O_6$	50.94	6.41	6.60
					(50.64	6.30	6.28)
21	Foam		17	$C_{18}H_{27}F_3N_2O_6$	50.94	6.41	6.60
					(50.41	6.40	6.33)
22	152—153	EtOH	18	$C_{22}H_{35}F_3N_2O_6$	54.99	7.34	5.83
					(54.88	7.31	5.78)
23	179.5—181	EtOH	22	$C_{23}H_{31}F_3N_2O_6$	56.55	6.40	5.73
					(56.80	6.56	5.82)
24	163—165	EtOH	24	$C_{14}H_{19}F_3N_2O_6$	45.66	5.20	7.61
					(45.36	5.28	7.63)
25	161—164.5	EtOH	33	$C_{15}H_{21}F_3N_2O_6$	47.12	5.53	7.33
					(46.89	5.65	7.40)
26	Foam		12	$C_{19}H_{21}F_3N_2O_6$	53.02	4.91	6.51
					(52.85	4.91	6.56)

TABLE VI. (continued)

Compd.	mp (°C)	Recryst. solvent	Yield (%)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
27	127 (dec.)	EtOH–pet. ether	32	$C_{20}H_{23}F_3N_2O_6$	54.05 (54.23	5.22 5.34	6.30 6.33)
28	121—123	EtOH–pet. ether	36	$C_{21}H_{25}F_3N_2O_6$	55.02 (55.14	5.50 5.61	6.11 6.16)
29	149—153	$CHCl_3$–pet. ether	22	$C_{17}H_{25}F_3N_2O_6$	49.75 (49.76	6.14 6.11	6.83 6.77)
30	Oil		20	$C_{28}H_{31}F_3N_2O_7$	59.57 (59.73	5.53 5.61	4.96 4.80)
31	163.5—166	EtOH	27	$C_{19}H_{21}F_3N_2O_6$	53.03 (52.94	4.92 5.08	6.51 6.55)
32	Foam		22	$C_{19}H_{21}F_3N_2O_6$	53.03 (53.10	4.92 5.02	6.51 6.49)
33	Oil		12	$C_{36}H_{47}F_3N_2O_7$	63.89 (64.00	7.00 7.14	4.14 4.12)
34	147—149	Benzene–EtOH	18	$C_{23}H_{29}F_3N_2O_6 \cdot 1/2\,H_2O$	55.75 (55.67	6.10 6.03	5.65 5.95)
35	Foam		13	$C_{23}H_{29}F_3N_2O_6$	56.79 (56.68	6.01 6.08	5.76 5.63)
36	164	EtOH–pet. ether	26	$C_{19}H_{21}F_3N_2O_6$	53.03 (52.79	4.92 5.12	6.51 6.74)
37	145—148	EtOH	12	$C_{20}H_{23}F_3N_2O_6$	54.05 (53.78	5.22 5.13	6.30 6.42)
38	Foam		17	$C_{21}H_{25}F_3N_2O_6 \cdot 1/4\,H_2O$	54.48 (54.65	5.55 5.58	6.05 6.06)
39	Foam		15	$C_{21}H_{25}F_3N_2O_6$	55.02 (54.80	5.50 5.56	6.11 5.88)
40	135.5—137	Benzene–$CHCl_3$	22	$C_{22}H_{27}F_3N_2O_6$	55.93 (55.95	5.76 5.92	5.93 5.93)
41	Foam		17	$C_{22}H_{27}F_3N_2O_6$	55.93 (55.83	5.76 5.81	5.93 5.78)
42	148—151	EtOH–pet. ether	12	$C_{19}H_{21}F_3N_2O_6$	53.02 (52.74	4.92 5.21	6.51 6.74)
43	Foam		11	$C_{20}H_{23}F_3N_2O_6$	54.05 (53.78	5.22 5.24	6.30 6.10)

the methods cited.

2′-Deoxy-3′,5′-di-*O*-(tetrahydro-2-furyl)-5-trifluoromethyluridine (1)——*p*-Toluenesulfonic acid (80 mg) was added to a suspension of F_3Thd (20 g, 0.067 mol) and 2,3-dihydrofuran (16.8 g, 0.24 mol) in dioxane (160 ml), and the mixture was stirred for 0.5 h at room temperature. The mixture was neutralized with 0.1 N C_2H_5ONa and concentrated. The residue was extracted with $CHCl_3$, then the extract was washed with water, dried over Na_2SO_4 and concentrated. The residue was crystallized from EtOH, giving 19.6 g of **1** in 67% yield. NMR (DMSO-d_6): 11.90 (1H, s, N^3-H), 8.12 (1H, s, H-6), 5.95 (1H, t, H-1′), 5.16 (2H, d, H-2 of tetrahydrofuryl group), 4.16 (2H, m, H-3′, 4′), 3.86 (6H, br, H-5′ and H-5 of tetrahydrofuryl group), 2.30 (2H, t, H-2′).

2′-Deoxy-5′-*O*-(tetrahydro-2-pyranyl)-5-trifluoromethyluridine (2)——*p*-Toluenesulfonic acid (60 mg) was added to a suspension of F_3Thd (2 g, 6.7 mmol) and 2-methoxytetrahydropyran (2.4 g, 20.4 mmol), and the mixture was stirred for 4 h at 60 °C, then neutralized with 0.1 N C_2H_5ONa and concentrated. The residue was extracted with $CHCl_3$, then the extract was washed with water, dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$: EtOH = 10 : 1, v/v) and the eluate was concentrated to dryness to afford **2** (0.46 g, 18%) as a foam. NMR (DMSO-d_6): 11.90 (1H, s, N^3-H), 8.14 (1H, d, H-6), 6.06 (1H, t, H-1′), 5.35 (1H, d, HO-3′), 4.60 (1H, s, H-2 of tetrahydropyranyl group), 4.24 (1H, m, H-3′), 4.04 (1H, m, H-4′), 2.21 (2H, t, H-2′).

5′-*O*-Benzyloxymethyl-2′-deoxy-5-trifluoromethyluridine (13)——*p*-Toluenesulfonic acid (120 mg) was added to a suspension of F_3Thd (2 g, 6.7 mmol) and dibenzyloxymethane (6.2 g, 27.2 mmol), and the mixture was stirred for 3 h

at 60 °C, then neutralized with 0.1 N C_2H_5Na and concentrated. The residue was extracted with $CHCl_3$. The extract was washed with water and concentrated. The residue was purified by silica-gel column chromatography ($CHCl_3$: EtOH = 10 : 1, v/v) and the product was crystallized from EtOH, giving 680 mg of **13** in 24% yield. NMR (DMSO-d_6): 11.88 (1H, s, N^3-H), 8.40 (1H, s, H-6), 6.09 (1H, t, H-1'), 5.40 (1H, d, HO-3'), 4.76 (2H, s, formalic methylene), 4.54 (2H, s, benzylic methylene), 4.26 (1H, m, H-3'), 3.97 (1H, q, H-4'), 2.31 (2H, t, H-2'). Other fractions of the eluate gave 560 mg (15%) of **12** and 655 mg (23%) of **14**.

Compounds **3**—**11** and **15**—**43** were synthesized similarly. The structures of these compounds were confirmed by the elemental analyses as well as by ^{1}H-NMR measurements (Table VI).

Antitumor Activity Test——Five-week-old male ICR mice (Japan Clea Inc., Tokyo, Japan) were inoculated subcutaneously in the axillary region with 5×10^6 sarcoma 180 cells, and test compounds were given orally once a day for 7 consecutive days beginning 24 h after inoculation of the tumor cells. Groups of seven mice were used for each dose and the test compounds were suspended in 0.5% carboxymethylcellulose (CMC) solution containing 0.1% Tween 80. On day 10, the tumors were excised and weighed. The inhibitory effects of test compounds were calculated from the ratio of the tumor weight in the test group to that in the control group.

Inhibitory effects of F_3Thd derivatives on the growth of solid tumor S 180 are shown in terms of the ED_{50} values in Tables I—V.

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Sulfur-Containing Acylamino Acids. I. Syntheses and Angiotensin I Converting Enzyme-Inhibitory Activities of Sulfur-Containing *N*-Mercaptoalkanoyl Amino Acids

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N-Mercaptoalkanoyl derivatives of sulfur-containing amino acids were synthesized and examined for inhibitory effects on angiotensin I converting enzyme (ACE) extracted from rabbit lung. Inhibition of ACE was determined by means of a spectrometric assay with hippuryl-L-histidyl-L-leucine as a substrate. Among the synthesized sulfur-containing compounds, *N*-(2-benzyl-3-mercaptopropanoyl)-*S*-methyl-L-cysteine (**13a**) and *N*-(2-benzyl-3-mercaptopropanoyl)-*S*-ethyl-L-cysteine (**13c**) showed the most potent inhibitory effects on ACE activity. The IC_{50} values of **13a** and **13c** on ACE activity were 0.028 and 0.020 μM, respectively.

Keywords——angiotensin I converting enzyme (ACE); ACE inhibitor; antihypertensive agent; sulfur-containing *N*-mercaptoalkanoyl amino acid; structure-activity relationship

Introduction

The renin–angiotensin system (RAS) is a multiregulated proteolytic cascade that produces two potent pressor and aldosteronogenic peptides; an octapeptide, angiotensin II, and a heptapeptide, angiotensin III.[1] Although the exact role that the RAS plays in the maintenance of normal blood pressure is unclear, it has been reported that pharmacological interruption of the RAS can lower blood pressure in a majority of hypertensive patients.[2] Specificially, inhibitors of angiotensin I converting enzyme (ACE) have been receiving much attention as antihypertensive drugs.

Since Ondetti *et al.* developed captopril in 1977,[3] many ACE inhibitors[4] including MK-421 have been synthesized. Most of these agents, as well as the naturally occurring ACE inhibitors, have proline, a cyclic amino acid, as the COOH-terminal residue. As part of a program on the development of novel ACE inhibitors, we synthesized *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids, as shown in Fig. 1, and tested them for ability to inhibit ACE.

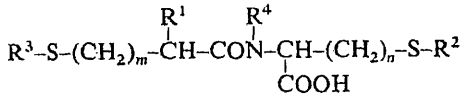


Fig. 1

Chemistry

The compounds listed in Table I were prepared by standard synthetic techniques (Chart

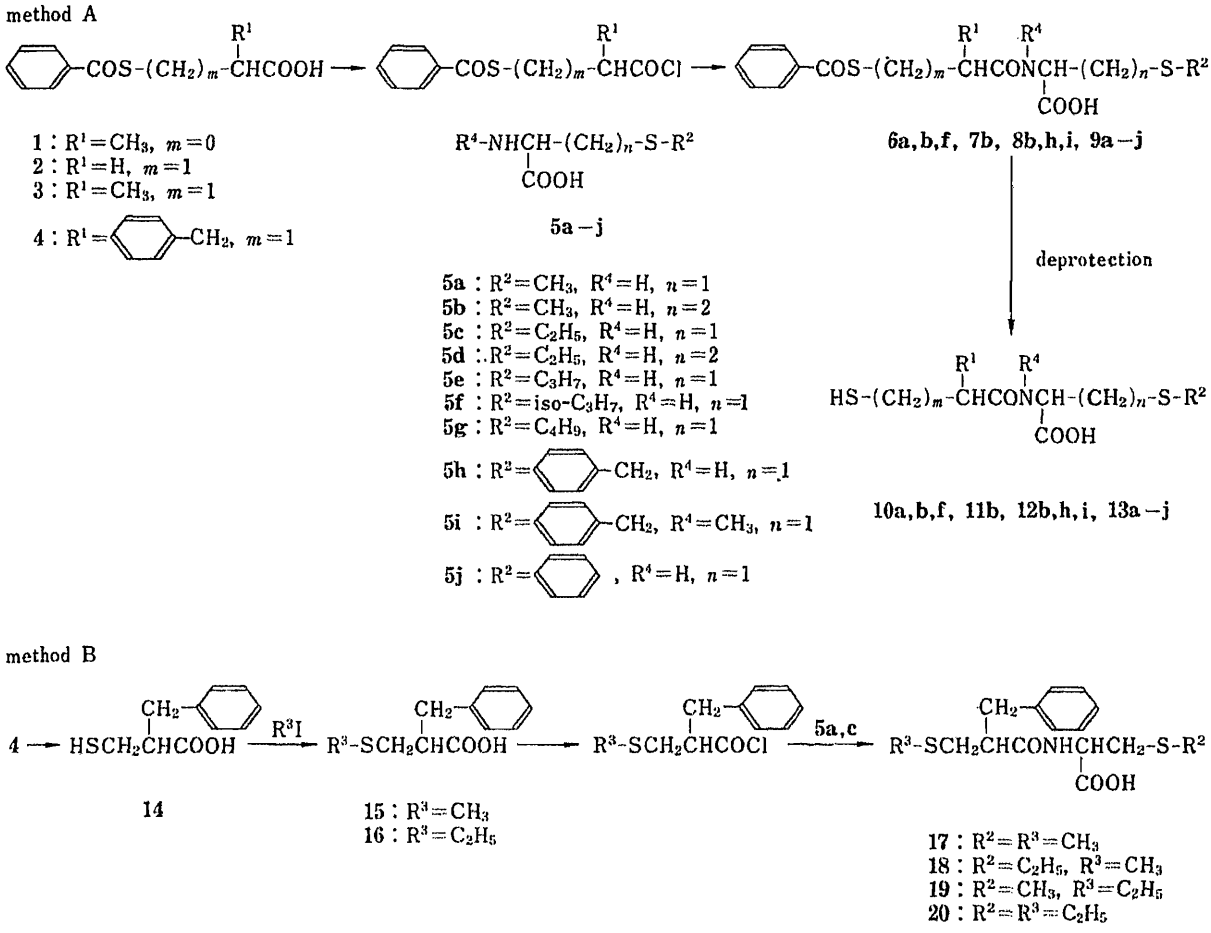


Chart 1

1). *N*-Mercaptoalkanoyl amino acids (**10**—**13**) were prepared by condensation of sulfur-containing amino acids (**5**) with (benzoylthio)alkanoyl chlorides [obtained by treatment of the corresponding acids (**1**—**4**) with $SOCl_2$], followed by debenzoylation with aqueous ammonia (method A). *N*-(Alkylthio)alkanoylamino acids (**17**—**20**) were prepared by the following method (method B). Mercaptocarboxylic acids (**14**) obtained by deprotection of **4** were alkylated with alkyl iodide, and then treated with $SOCl_2$. The acid chlorides thus obtained were condensed with amino acids (**5a**, **c**) to give **17**—**20**.

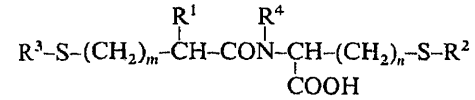
Biological Results and Discussion

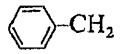
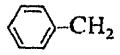
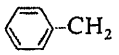
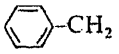
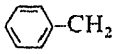
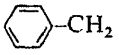
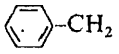
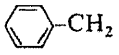
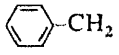
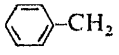
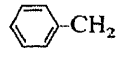
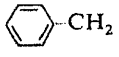
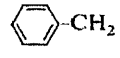
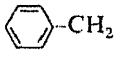
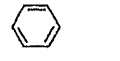
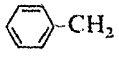
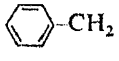
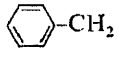
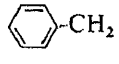
We evaluated the inhibitory effects of *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids on ACE activity *in vitro*. The IC_{50} values of test compounds on ACE are summarized in Table I.

In Fig. 1, the sulfur-containing amino acid moiety of the compounds with $n=1$ is an L-cysteine derivative. The amino acid moiety in the case of the compounds with $n=2$ is DL-methionine or DL-ethionine (for **13b**, L-configuration).

The ACE-inhibitory activity of compounds with *S*-methylcysteine (**13a**) and *S*-ethylcysteine (**13c**) as the sulfur-containing amino acid moiety was more potent than that of compounds with methionine (**13b**) and ethionine (**13d**). On the other hand, the inhibitory action of **12b** ($m=1$) was more potent than that of **10b** ($m=0$). These results suggest that the structure–activity correlation concerning the length of the carbon chain is as follows:

TABLE I. Structure and Inhibitory Activity



Compound No.	m	n	R^1	R^2	R^3	R^4	IC_{50} (μM)
10a[a]	0	1	CH_3	CH_3	H	H	0.46
10b[b]	0	2	CH_3	CH_3	H	H	0.63
10f[a]	0	1	CH_3	iso-C_3H_7	H	H	3.5
11b[c]	1	2	H	CH_3	H	H	0.62
12b[b]	1	2	CH_3	CH_3	H	H	0.28
12h[a]	1	1	CH_3		H	H	1.0
12i[a]	1	1	CH_3		H	CH_3	>10
13a[d]	1	1		CH_3	H	H	0.028
13b[a]	1	2		CH_3	H	H	0.042
13c[d]	1	1		C_2H_5	H	H	0.020
13d[b]	1	2		C_2H_5	H	H	0.21
13e[a]	1	1		C_3H_7	H	H	0.39
13f[a]	1	1		iso-C_3H_7	H	H	0.56
13g[a]	1	1		C_4H_9	H	H	0.39
13h[a]	1	1			H	H	0.15
13i[a]	1	1			H	CH_3	>10
13j[a]	1	1			H	H	0.83
17[a]	1	1		CH_3	CH_3	H	>100
18[a]	1	1		C_2H_5	CH_3	H	>100
19[a]	1	1		CH_3	C_2H_5	H	>100
20[a]	1	1		C_2H_5	C_2H_5	H	>100
Captopril							0.028

a) Mixtures of diastereomers, whose ratio could not be determined, as indicated by thin layer chromatography and nuclear magnetic resonance (NMR). *b*) Mixtures of two diastereomeric pairs of enantiomers. *c*) Racemate. *d*) **13a** and **13c** were mixtures of diastereomers in ratios approximately 1 : 0.7 and 1 : 0.8, respectively, as indicated by NMR.

$(n=1)>(n=2)$, $(m=1)>(m=0)$.

Replacement of the methyl group (**12h**) with benzyl (**13h**) at the R^1-position led to enhancement of the inhibitory potency. In addition, based on the inhibitory activities of compounds **11b**, **12b** and **13b** on ACE activity, the structure–activity correlation for substitution at the R^1-position was concluded to be as follows: benzyl > CH_3 > H. In compounds having benzyl at the R^1-position, the effectiveness of R^2 substituents on inhibitory activity was in the order CH_3 (**13a**), C_2H_5 (**13c**) > benzyl (**13h**) > C_3H_7 (**13e**), C_4H_9

(**13f**), iso-C_3H_7 (**13g**)>phenyl (**13j**). The above relation was also observed in the cases of compounds **10a** (CH_3) and **10f** (iso-C_3H_7) having methyl at the R^1-position. The alkylation of SH caused complete loss of the inhibitory action (**17**—**20**). Introduction of a methyl group on NH markedly lowered the ACE-inhibitory action (**12i**, **13i**).

Thus, compounds which possess benzyl at the R^1-position, methyl or ethyl at the R^2-position and hydrogen at the R^3- and R^4-positions showed potent ACE-inhibitory action. Among them, compounds **13a** and **13c** were the most potent inhibitors, and their IC_{50} values were 0.028 and 0.020 μM, respectively. The ACE-inhibitory activities of these compounds were found to be almost equal to that of captopril (IC_{50} value, 0.028 μM).

Experimental

Melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded with a JASCO IR-810 spectrometer. Optical rotations were measured with a JASCO DIP-360 polarimeter.

***N*-(2-Benzyl-3-mercaptopropanoyl)-*S*-ethyl-L-cysteine (13c)**——A mixture of 3-(benzoylthio)-2-benzylpropanoic acid (**4**) and $SOCl_2$ (8 ml) was stirred at 30—40 °C for 2 h. Evaporation of the excess $SOCl_2$ gave 3-(benzoylthio)-2-benzylpropanoyl chloride. The acid chloride was used without purification in the following reaction: 25.5 g (0.08 mol) of the acid chloride and 80 ml of 1 N NaOH were added dropwise to a solution of *S*-ethyl-L-cysteine (**5c**) (11.9 g, 0.08 mol) in 1 N NaOH (80 ml) and acetone (160 ml) at 5—10 °C. After being stirred at room temperature overnight, the mixture was acidified with conc. HCl and extracted with EtOAc. The extract was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 28.0 g (81%) of *N*-[3-(benzoylthio)-2-benzylpropanoyl]-*S*-ethyl-L-cysteine (**9c**), mp 76—79 °C. $[\alpha]_D^{25}$ −8.4° (c=2, EtOH). *Anal.* Calcd for $C_{22}H_{25}NO_4S_2$: C, 61.23; H, 5.84; N, 3.25. Found: C, 61.26; H, 5.84; N, 3.30.

A solution of **9c** (27.6 g, 0.064 mol) in MeOH (30 ml) was added to 28% aqueous ammonia (40 ml). After being stirred for 2 h at room temperature, the reaction mixture was evaporated *in vacuo* and the residue was diluted with water and extracted with EtOAc to remove benzamide. The aqueous layer was acidified with conc. HCl, saturated with NaCl and extracted with EtOAc. This EtOAc extract was washed with saturated NaCl solution, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 13.0 g (62%) of **13c**, mp 64—70 °C. $[\alpha]_D^{25}$ −31.6° (c=2.0, EtOH). IR (KBr): 2550 (SH) cm^{-1}. *Anal.* Calcd for $C_{15}H_{21}NO_3S_2$: C, 55.02; H, 6.46; N, 4.28. Found: C, 55.04; H, 6.57; N, 4.35.

***N*-(2-Benzyl-3-mercaptopropanoyl)-*S*-methyl-L-cysteine (13a)**——This compound was similarly prepared from **4** and *S*-methyl-L-cysteine (**5a**) *via* intermediate *N*-[3-(benzoylthio)-2-benzylpropanoyl]-*S*-methyl-L-cysteine (**9a**) (dicyclohexylamine (DCHA) salt of **9a**: mp 127—129 °C. *Anal.* Calcd for $C_{21}H_{23}NO_4S_2 \cdot C_{12}H_{23}N$: C, 66.18; H, 7.74; N, 4.68. Found: C, 66.09; H, 7.83; N, 4.70).

13a: Oil. IR (neat): 2550 (SH) cm^{-1}. DCHA salt: mp 152—154 °C. *Anal.* Calcd for $C_{14}H_{19}NO_3S_2 \cdot C_{12}H_{23}N$: C, 63.12; H, 8.56; N, 5.66. Found: C, 62.84; H, 8.61; N, 5.62.

The following compounds (**13b, d**—**j**) were prepared in a similar manner.

13b: mp 144—147 °C (EtOAc) (lit.[5] mp 137—138 °C). $[\alpha]_D^{25}$ −29.1° (c=2, EtOH). *Anal.* Calcd for $C_{15}H_{21}NO_3S_2$: C, 55.02; H, 6.46; N, 4.28. Found: C, 55.01; H, 6.55; N, 4.24.

13d: mp 124—126 °C. $[\alpha]_D^{25}$ +0.0° (c=2, EtOH). *Anal.* Calcd for $C_{16}H_{23}NO_3S_2$: C, 56.28; H, 6.78; N, 4.10. Found: C, 56.13; H, 6.93; N, 4.10.

13e: Oil. DCHA salt: mp 121—125 °C. *Anal.* Calcd for $C_{16}H_{23}NO_3S_2 \cdot C_{12}H_{23}N$: C, 64.33; H, 8.87; N, 5.36. Found: C, 64.05; H, 8.98; N, 5.45.

13f: mp 142.5—147.5 °C. $[\alpha]_D^{25}$ +8.48° (c=2, EtOH). *Anal.* Calcd for $C_{16}H_{23}NO_3S_2 \cdot C_{12}H_{23}N$: C, 64.23; H, 8.87; N, 5.36. Found: C, 64.03; H, 9.04; N, 5.30.

13g: Oil. $[\alpha]_D^{25}$ −24.3° (c=2, EtOH). DCHA salt: mp 121—138 °C. *Anal.* Calcd for $C_{17}H_{25}NO_3S_2 \cdot C_{12}H_{23}N$: C, 64.88; H, 9.01; N, 5.22. Found: C, 64.63; H, 8.99; N, 5.28.

13h: Oil. $[\alpha]_D^{25}$ +3.5° (c=1, $CHCl_3$). IR (neat): 2570 (SH) cm^{-1}. DCHA salt: mp 139—144 °C. *Anal.* Calcd for $C_{20}H_{23}NO_3S_2 \cdot C_{12}H_{23}N$: C, 67.32; H, 8.12; N, 4.91. Found: C, 67.11; H, 8.12; N, 4.84.

13i: Oil. $[\alpha]_D^{25}$ −32.8° (c=1, $CHCl_3$). DCHA salt: mp 139—143 °C. *Anal.* Calcd for $C_{21}H_{25}NO_3S_2 \cdot C_{12}H_{23}N$: C, 67.77; H, 8.27; N, 4.79. Found: C, 67.48; H, 8.31; N, 4.66.

13j: mp 110—112 °C. $[\alpha]_D^{25}$ +10.9° (c=1, $CHCl_3$). IR (KBr): 2570 (SH) cm^{-1}. *Anal.* Calcd for $C_{19}H_{21}NO_3S_2$: C, 60.77; H, 5.64; N, 3.73. Found: C, 61.00; H, 5.70; N, 3.77.

The following compounds (**10a, b, f**) were prepared from 2-(benzoylthio)propanoic acid (**1**) in the same manner as described for **13c**.

10a: mp 80—81 °C. $[\alpha]_D^{25}$ +14.5° (c=2, EtOH). *Anal.* Calcd for $C_7H_{13}NO_3S_2$: C, 37.65; H, 5.87; N, 6.27. Found:

C, 37.57; H, 5.93; N, 6.25.

10b: mp 119—123 °C. $[\alpha]_D^{25}$ +0.1° (c=2, EtOH). *Anal.* Calcd for $C_8H_{15}NO_3S_2$: C, 40.49; H, 6.37; N, 5.90. Found: C, 40.57; H, 6.38; N, 6.03.

10f: Gummy solid. $[\alpha]_D^{25}$ −3.4° (c=2, EtOH). DCHA salt: mp 169—172 °C. *Anal.* Calcd for $C_9H_{17}NO_3S_2 \cdot C_{12}H_{23}N$: C, 58.29; H, 9.32; N, 6.47. Found: C, 58.15; H, 9.37; N, 6.48.

Compound **11b** was prepared from 3-(benzoylthio)propanoic acid (**2**) in the same manner as described for **13c**.

11b: mp 82—84 °C. $[\alpha]_D^{25}$ +0.1° (c=2, EtOH). *Anal.* Calcd for $C_8H_{15}NO_3S_2$: C, 40.49; H, 6.37; N, 5.90. Found: C, 40.39; H, 6.38; N, 6.00.

The following compounds (**12b, h, i**) were similarly prepared from 3-(benzoylthio)-2-methylpropanoic acid (**3**).

12b: mp 70—72 °C. *Anal.* Calcd for $C_9H_{17}NO_3S_2$: C, 43.00; H, 6.82; N, 5.57. Found: C, 43.09; H, 6.94; N, 5.53.

12h: Oil. $[\alpha]_D^{25}$ −39.6° (c=2, EtOH). DCHA salt: *Anal.* Calcd for $C_{14}H_{19}NO_3S_2 \cdot C_{12}H_{23}N$: C, 63.12; H, 8.56; N, 5.66. Found: C, 62.89; H, 8.75; N, 5.51.

12i: Oil. $[\alpha]_D^{25}$ −84.5° (c=2, EtOH). DCHA salt: *Anal.* Calcd for $C_{15}H_{21}NO_3S_2 \cdot C_{12}H_{23}N \cdot H_2O$: C, 61.56; H, 8.80; N, 5.32. Found: C, 61.67; H, 8.83; N, 5.34.

***N*-[2-Benzyl-3-(ethylthio)propanoyl]-*S*-methyl-L-cysteine (19)**——Sodium (4.6 g, 0.2 mol) was added to a solution of 2-benzyl-3-mercaptopropanoic acid (**14**) (19.6 g, 0.1 mol) in absolute EtOH (180 ml). When the last piece of sodium was about to disappear, ethyl iodide (15.6 g, 0.1 mol) was added with stirring. After 1 h the reaction mixture was concentrated *in vacuo*. The residue was dissolved in water (200 ml) and extracted with Et_2O. The aqueous layer was acidified with HCl, and extracted with EtOAc. The extract was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 19.0 g (84%) of 2-benzyl-3-(ethylthio)propanoic acid (**16**) as a colorless oil.

A solution of this oil (**16**) (9.0 g, 0.04 mol) and $SOCl_2$ (8 ml) was stirred at room temperature overnight. Evaporation of the excess $SOCl_2$ gave 2-benzyl-3-(ethylthio)propanoyl chloride as a clear oil. This crude acid chloride was used without purification in the following reaction. 2-Benzyl-3-(ethylthio)propanoyl chloride (9.71 g, 0.04 mol) and 1 N NaOH (40 ml) were added dropwise to a solution of **5a** (5.41 g, 0.04 mol) in 1 N NaOH (40 ml) and Et_2O (40 ml) at 5—10 °C. The mixture was stirred overnight at room temperature, then the aqueous layer was separated with HCl and extracted with EtOAc. The extract was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 9.9 g (72%) of **19**, mp 113—117 °C (Et_2O). $[\alpha]_D^{25}$ −30.8° (c=2, EtOH). *Anal.* Calcd for $C_{16}H_{23}NO_3S_2$: C, 56.28; H, 6.79; N, 4.10. Found: C, 56.03; H, 6.86; N, 4.15.

The corresponding *N*-[2-benzyl-3-(alkylthio)propanoyl] compounds (**17, 18, 20**) were similarly prepared.

17: mp 82—87 °C. $[\alpha]_D^{25}$ −25.1° (c=2, EtOH). *Anal.* Calcd for $C_{15}H_{21}NO_3S_2$: C, 55.02; H, 6.46; N, 4.28. Found: C, 55.05; H, 6.59; N, 4.30.

18: mp 81—92 °C. $[\alpha]_D^{25}$ −22.5° (c=2, EtOH). *Anal.* Calcd for $C_{16}H_{23}NO_3S_2$: C, 56.28; H, 6.79; N, 4.10. Found: C, 56.52; H, 6.89; N, 4.11.

20: mp 100—105 °C. $[\alpha]_D^{25}$ −23.7° (c=2, EtOH). *Anal.* Calcd for $C_{17}H_{25}NO_3S_2$: C, 57.43; H, 7.09; N, 3.94. Found: C, 57.41; H, 7.24; N, 3.97.

Biological Methods——ACE was partially purified from male rabbit lung by a modification of the method of Soffer *et al.*[6] The *in vitro* ACE-inhibitory activities of *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids were determined with hippuryl-histidyl-leucine (HHL) as a substrate, according to the method of Cushman and Cheung.[7] ACE, HHL and test samples were dissolved in 0.1 M potassium phosphate buffer (pH 8.3). A mixture of 0.05 ml of ACE solution and 0.1 ml of test sample solution was preincubated at 37 °C for 5 min, then 0.1 ml of 0.1 M HHL was added. After incubation at 37 °C for 30 min, the enzyme reaction was stopped by the addition of 0.25 ml of 1 N HCl. Hippuric acid (HA) generated from the substrate was extracted with 1.5 ml of EtOAc, then 1 ml of EtOAc was evaporated off at 50 °C. HA was redissolved in 1 ml of 1 M NaCl, and the absorbance at 228 nm was measured. The amount of HA generated from the substrate was estimated from the calibration plot for HA (obtained by the same procedure). Inhibitory activity was expressed as micromolar concentration of the test compounds producing 50% inhibition of ACE activity (IC_{50}).

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Sulfur-Containing Acylamino Acids. II. Syntheses and Angiotensin I Converting Enzyme-Inhibitory Activities of *N*-Mercaptoalkanoyl-*S*-ethyl-L-cysteine[1)]

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N-Mercaptoalkanoyl derivatives of sulfur-containing amino acids were synthesized as candidate angiotensin I converting enzyme (ACE) inhibitors. Among them, *N*-[3-mercapto-2-(4-methoxybenzyl)propanoyl]-*S*-ethyl-L-cysteine (**5d**) was found to be the most potent inhibitor of ACE, with an IC_{50} value of 0.045 μM. The maximum hypotensive effect of this compound was almost equal to that of captopril in anesthetized rats.

Keywords——angiotensin I converting enzyme (ACE); ACE inhibitor; antihypertensive agent; *N*-mercaptoalkanoyl-*S*-ethyl-L-cysteine; structure–activity relationship

Introduction

We previously synthesized *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids, and reported that compound **8**, having benzyl at the R^1 position, ethyl at the R^2 position and hydrogen at the R^3 and R^4 positions in Fig. 1, was a rather potent inhibitor of angiotensin I converting enzyme (ACE).[1)] As part of an effort to develop active ACE inhibitors, we synthesized additional *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids. We examined the effects of these compounds, which showed potent ACE-inhibitory action *in vitro*, on the pressor responses to angiotensin I (AI) and the depressor responses to bradykinin (BK) in anesthetized rats.

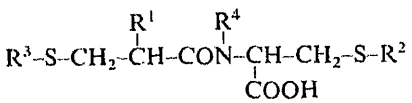


Fig. 1

Chemistry

Compounds listed in Table I were prepared by standard techniques (Chart 1). 3-(Acylthio)-2-alkylpropanoic acids (**1**, **2**), prepared by Michael addition of thiobenzoic acid to α-alkylacrylic acids obtained by the method of Mannich and Ganz,[2)] were treated with $SOCl_2$ to give the corresponding acid chlorides (**3**), which were condensed with *S*-ethyl-L-cysteine (**4**) to give the corresponding *N*-(acylthio)alkanoylamino acids (**6**, **7**). Removal of the benzoyl group of **7b—k** was carried out with aqueous ammonia to give the corresponding *N*-

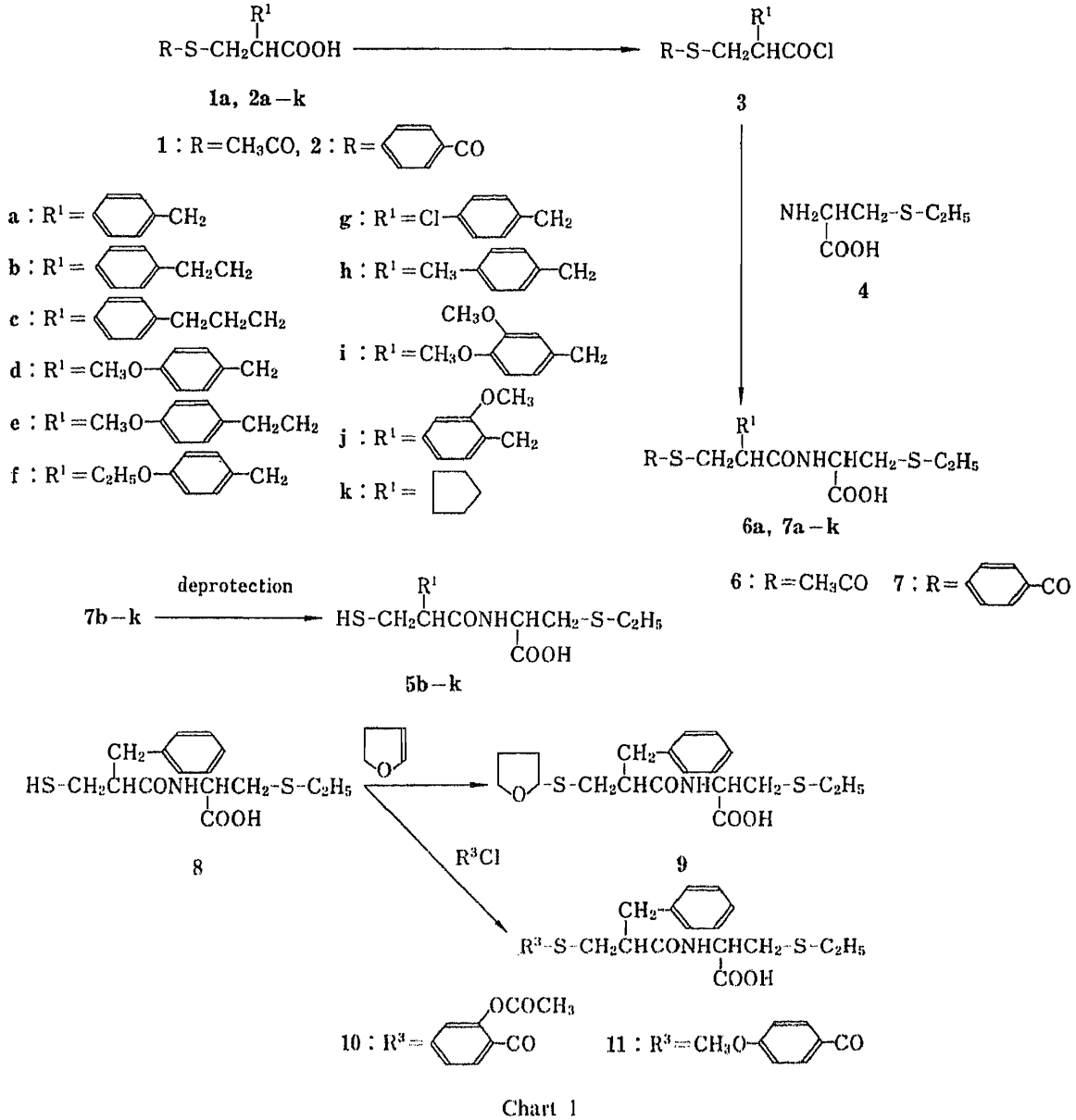


Chart 1

mercaptoalkanoyl-*S*-ethyl-L-cysteines (**5b**—**k**).

The (tetrahydrofuryl)thio compound (**9**) was obtained by addition of the mercapto compound (**8**) to 2,3-dihydrofuran in the presence of *p*-toluenesulfonic acid. *N*-[3-(Substituted benzoylthio)-2-benzylpropanoyl]-*S*-ethyl-L-cysteines (**10**, **11**) were prepared by reaction of **8** with the corresponding substituted benzoyl chlorides. Compounds **12** and **13** listed in Table III were prepared by condensation of **5d** with *N*-benzoyl-D-amino acids in the presence of *N*,*N*′-carbonyldiimidazole.

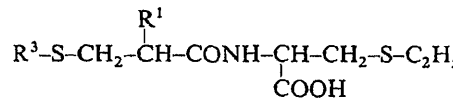
Biological Results and Discussion

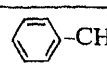
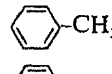
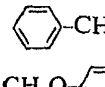
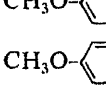
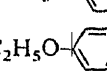
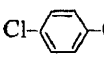
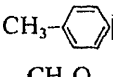
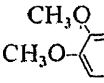
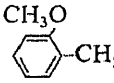
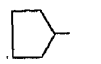
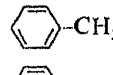
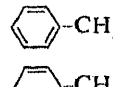
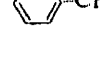
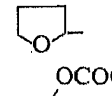
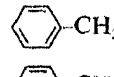
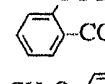
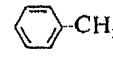
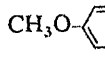
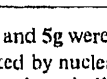
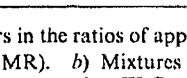
In Vitro ACE Inhibitory Activity

We evaluated the *in vitro* ACE-inhibitory activities of the synthesized *N*-mercaptoalkanoyl derivatives of sulfur-containing amino acids; the IC_{50} values are summarized in Table I.

As regards the length of methylene chain in the phenylalkyl moiety at the R^1 position,

TABLE I. Structure and Inhibitory Activity of Test Compounds



Compound No.	R^1	R^3	IC_{50} (μM)
8[a]		H	0.050
5b[b]		H	0.17
5c[b]		H	1.0
5d[b]		H	0.045
5e[b]		H	0.070
5f[b]		H	0.045
5g[a]		H	0.045
5h[c]		H	0.42
5i[c]		H	0.073
5j[b]		H	0.80
5k[b]		H	5.0
6a[b]		CH_3CO-	0.76
7a[c]			0.050
9[b]			0.092
10[b]			>1.0
11[c]			0.28
Captopril			0.042

a) Compounds **8** and **5g** were mixtures of diastereomers in the ratios of approximately 1 : 0.8 and 1 : 0.7, respectively, as indicated by nuclear magnetic resonance (NMR). *b*) Mixtures of distereomers, whose ratio could not be determined, as indicated by thin-layer chromatography (TLC) and NMR. *c*) Mixtures of diastereomers in approximately equal ratio.

CH_2 (compound **8**) resulted in higher activity than $(CH_2)_2$ (compound **5b**) or $(CH_2)_3$ (compound **5c**). The activities of **5b** and **5c** were 1/3 and 1/20 of that of **8**, respectively. Compounds having a *p*-methoxybenzyl (**5d**), *p*-methoxyphenethyl (**5e**), *p*-ethoxybenzyl (**5f**), *p*-chlorobenzyl (**5g**) or *m*,*p*-dimethoxybenzyl (**5i**) group at the R^1 position showed the same inhibitory action as **8** *in vitro*. However, introduction of a *p*-methyl (**5h**) or an *o*-methoxy (**5j**) group into the benzyl moiety of compound **8** led to a decrease of activity. Compound **5k**, having a cyclopentyl group at the R^1 position, showed little potency.

We previously reported that compounds having hydrogen at the R^3 position showed

potent inhibitory action towards ACE, but alkylation at this position caused complete loss of the inhibitory activity. To investigate the effects of replacement of the R^3 moiety with substituents other than alkyl, a number of derivatives were prepared. The ACE-inhibitory activity of the compound possessing a benzoyl (**7a**) or tetrahydrofuryl (**9**) group was almost equal to that of compound **8**, whereas replacement of the hydrogen with an acetyl (**6a**), *o*-acetoxybenzoyl (**10**) or *p*-methoxybenzoyl (**11**) group lowered the activity.

In Vivo ACE-Inhibitory Action

Table II shows the *in vivo* results obtained with selected ACE inhibitors. Compound **5d**, which was the most potent ACE inhibitor among the compounds tested in the present study, caused a significant reduction (26 mmHg) of arterial blood pressure in anesthetized rats. The arterial blood pressure level returned to the control level within 10 min after administration. On the other hand, the hypotensive action of captopril reached the maximum at 1 to 3 min after administration and lasted for at least 1 h. The injection of compound **5d** resulted in inhibition of the pressor responses to AI and potentiation of the depressor responses to BK, although these actions of the compound were of short duration compared with those induced by captopril.

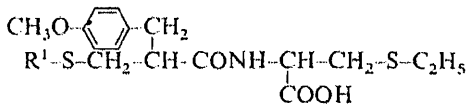
It was reported that the introduction of a cyclohexanecarbonyl-D-alanyl[3] or benzoyl-D-phenylalanyl[4] group into the sulfhydryl moiety of captopril augments the duration of ACE-inhibitory action. Based on these findings, we synthesized compounds **12** and **13** which contain these groups in the sulfhydryl moiety of **5d**. However, these compounds were less potent than **5d** in terms of both ACE-inhibitory activity *in vitro* (Table III) and hypotensive action *in vivo*.

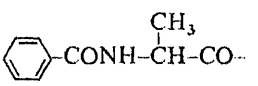
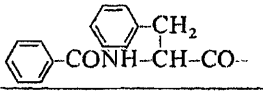
Table II. *In Vivo* Comparisons of Selected ACE Inhibitors Including Captopril

Compound No.	Δ mmHg[a]	AI (min)[b]	BK (min)[c]
5b	20	5	15
5d	26	25	35
7a	8	5	5
8	22	15	15
9	14	5	5
11	4	5	5
Captopril	30	60	60

a) Fall in blood pressure. *b*) Duration of inhibitory action on pressor responses to AI. *c*) Duration of potentiating action on depressor responses to BK.

Table III. Structure and Inhibitory Activity of Test Compounds



Compound No.	IC_{50} (μM)	R^1
12[a]	0.6	(D-) 
13[a]	0.6	(D-) 

a) Mixtures of two diastereomers.

Experimental

Melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded with a JASCO IR-810 spectrometer. Optical rotations were measured with a JASCO DIP-360 polarimeter.

***N*-[3-Mercapto-2-(4-methoxybenzyl)propanoyl]-*S*-ethyl-L-cysteine (5d)**——Diethyl 4-methoxybenzylmalonate[5] (72.9 g, 0.26 mol) was saponified with KOH (35 g, 0.63 mol) to give 52 g (89%) of 4-methoxybenzylmalonic acid. Then 35% aqueous formaldehyde (38 g) was added to a mixture of the malonic acid thus obtained (49.3 g, 0.22 mol) and 50% aqueous dimethylamine (20 g). After standing for 2 h, the mixture was diluted with water and filtered. The dimethylaminomethylated product (50.6 g, 0.18 mol) was dissolved in 1 N NaOH (180 ml) and the solution was refluxed for 2 h, during which time 10% H_2SO_4 was added as required to maintain neutrality. The reaction mixture was cooled, the pH was adjusted to 2 with 10% H_2SO_4, and the precipitated 2-(4-methoxybenzyl) acrylic acid was recovered by filtration (29.1 g, 84%).

A mixture of the above acrylic acid (9.61 g, 0.05 mol) and thiobenzoic acid (7.0 g, 0.05 mol) was heated on a steam bath for 2 h to give crude 3-(benzoylthio)-2-(4-methoxybenzyl)propanoic acid (**2d**).[6] A solution of this crude acid and $SOCl_2$ (8 ml) was stirred at 30—40 °C for 2 h. Evaporation of the excess $SOCl_2$ gave 3-(benzoylthio)-2-(4-methoxybenzyl)propanoyl chloride.

3-(Benzoylthio)-2-(4-methoxybenzyl)propanoyl chloride (17.4 g, 0.05 mol) and 1 N NaOH (50 ml) were added dropwise to a solution of *S*-ethyl-L-cysteine (**4**) (7.5 g, 0.05 mol) in 1 N NaOH (50 ml) and acetone (100 ml) at 5—10 °C. After being stirred at room temperature for 2 h, the mixture was acidified with conc. HCl and extracted with EtOAc. The extract was washed with water, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH) to give 11.7 g (51%) of *N*-[3-(benzoylthio)-2-(4-methoxybenzyl)-propanoyl]-*S*-ethyl-L-cysteine (**7d**). A solution of **7d** (11.7 g, 0.025 mol) in MeOH (30 ml) was added to 28% aqueous ammonia (25 ml). After being stirred for 2 h at room temperature, the reaction mixture was evaporated *in vacuo* and the residue was diluted with water and extracted with EtOAc to remove benzamide. The aqueous layer was acidified with conc. HCl, saturated with NaCl and extracted with EtOAc. This EtOAc extract was washed with saturated NaCl solution, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 4.5 g (50%) of **5d** as a colorless oil. $[\alpha]_D^{25}$ +9.87° (*c* = 0.46, $CHCl_3$). IR (neat): 2560 cm^{-1} (SH). For elemental analysis, this compound was converted to the dicyclohexylamine (DCHA) salt, mp 126—129 °C. *Anal.* Calcd for $C_{16}H_{23}NO_4S_2 \cdot C_{12}H_{23}N$: C, 62.42; H, 8.61; N, 5.20. Found: C, 62.19; H, 8.63; N, 5.18.

The following compounds (**5b**, **c**, **e**—**k**) were prepared in a similar manner.

5b: Oil, $[\alpha]_D^{25}$ −27.5° (*c* = 2, EtOH). DCHA salt: mp 140—143 °C. *Anal.* Calcd for $C_{16}H_{23}NO_3S_2 \cdot C_{12}H_{23}N$: C, 64.33; H, 8.87; N, 5.36. Found: C, 63.92; H, 9.11; N, 5.33.

5c: Oil, $[\alpha]_D^{25}$ −32.0° (*c* = 2, EtOH). DCHA salt: mp 129—132 °C. *Anal.* Calcd for $C_{17}H_{25}NO_3S_2 \cdot C_{12}H_{23}N$: C, 64.88; H, 9.01; N, 5.22. Found: C, 64.82; H, 9.18; N, 5.20.

5e: mp 84—88 °C. $[\alpha]_D^{25}$ +17.1° (*c* = 1, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{25}NO_4S_2$: C, 54.96; H, 6.78; N, 3.77. Found: C, 54.81; H, 6.85; N, 3.65.

5f: Oil, $[\alpha]_D^{25}$ +8.8° (*c* = 1, $CHCl_3$). DCHA salt: mp 166—168 °C. *Anal.* Calcd for $C_{17}H_{25}NO_4S_2 \cdot C_{12}H_{23}N$: C, 63.00; H, 8.75; N, 5.06. Found: C, 62.96; H, 8.89; N, 5.02.

5g: mp 81—83 °C. $[\alpha]_D^{25}$ +16.2° (*c* = 1, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{20}ClNO_3S_2$: C, 49.78; H, 5.57; N, 3.87. Found: C, 49.89; H, 5.55; N, 3.76.

5h: mp 88—91 °C. $[\alpha]_D^{25}$ +9.2° (*c* = 1, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{23}NO_3S_2$: C, 56.28; H, 6.79; N, 4.10. Found: C, 56.04; H, 6.87; N, 4.03.

5i: mp 130—133 °C. $[\alpha]_D^{25}$ −12.2° (*c* = 1, $CHCl_3$). *Anal.* Calcd for $C_{17}H_{25}NO_5S_2$: C, 52.69; H, 6.50; N, 3.61. Found: C, 52.49; H, 6.57; N, 3.60.

5j: Colorless gummy solid, $[\alpha]_D^{25}$ +32.5° (*c* = 1, $CHCl_3$). DCHA salt: mp 128—130 °C. *Anal.* Calcd for $C_{16}H_{23}NO_4S_2 \cdot C_{12}H_{23}N$: C, 62.42; H, 8.61; N, 5.20. Found: C, 62.40; H, 8.75; N, 5.17.

5k: mp 63—69 °C. $[\alpha]_D^{25}$ −23.8° (*c* = 2, EtOH). DCHA salt: *Anal.* Calcd for $C_{13}H_{23}NO_3S_2 \cdot C_{12}H_{23}N$: C, 61.69; H, 9.53; N, 5.75. Found: C, 61.40; H, 9.73; N, 5.67.

***N*-[3-(Acetylthio)-2-benzylpropanoyl]-*S*-ethyl-L-cysteine (6a)**——3-(Acetylthio)-2-benzylpropanoyl chloride (1.67 g, 6.5 mmol) and 1 N NaOH (6 ml) were added dropwise to a solution of **4** (0.90 g, 6 mmol) in 1 N NaOH (6 ml) and acetone (12 ml) at 5—10 °C. After being stirred at room temperature for 2 h, the mixture was acidified with HCl and extracted with EtOAc. The extract was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene–EtOAc) to give 1.86 g (84%) of **6a** as an oil. DCHA salt: mp 118—128 °C. *Anal.* Calcd for $C_{17}H_{23}NO_4S_2 \cdot C_{12}H_{23}N$: C, 63.24; H, 8.42; N, 5.09. Found: C, 62.99; H, 8.50; N, 5.11.

***N*-[2-Benzyl-3-(4-methoxybenzoylthio)propanoyl]-*S*-ethyl-L-cysteine (11)**——A solution of *p*-methoxybenzoyl chloride (2.05 g, 0.012 mol) in 5 ml of acetone and 1 N NaOH (12 ml) were added dropwise to a stirred solution of **8** (3.27 g, 0.01 mol) in 1 N NaOH (10 ml) and acetone with ice cooling. The resulting mixture was stirred at room temperature for 1 h, then acidified with HCl, and extracted with EtOAc. The extract was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed on silica gel ($CHCl_3$–MeOH) to give 2.6 g

(56%) of **11** as colorless crystals. mp 108—111 °C. $[\alpha]_D^{25}$ −3.5° (c=2, EtOH). *Anal.* Calcd for $C_{23}H_{27}NO_5S_2$: C, 59.85; H, 5.90; N, 3.30. Found: C, 59.67; H, 5.84; N, 3.06.

The corresponding *N*-[3-(substituted benzoylthio)-2-benzylpropanoyl]-*S*-ethyl-L-cysteine (**10**) was similarly synthesized.

10: Oil, DCHA salt: amorphous powder, no definite melting point. *Anal.* Calcd for $C_{24}H_{27}NO_6S_2 \cdot C_{12}H_{23}N \cdot 1/2H_2O$: C, 63.59; H, 7.56; N, 4.12. Found: C, 63.65; H, 7.49; N, 4.16.

***N*-[2-Benzyl-3-(tetrahydrofuran-2-ylthio)propanoyl]-*S*-ethyl-L-cysteine (9)**——A solution of **8** (660 mg, 2 mmol), 2,3-dihydrofuran (140 mg, 2 mmol) and a catalytic amount of *p*-toluenesulfonic acid in Et_2O (10 ml) was refluxed for 1 h. DCHA (370 mg, 2 mmol) was added and the reaction mixture was concentrated *in vacuo*. The residual oil was crystallized from Et_2O–hexane to give the DCHA salt of **9**, yield 500 mg. *Anal.* Calcd for $C_{19}H_{27}NO_4S_2 \cdot C_{12}H_{23}N \cdot H_2O$: C, 62.38; H, 8.78; N, 4.69. Found: C, 62.53; H, 8.62; N, 5.00.

***N*-[3-[*N*-Benzoyl-D-(phenylalanylthio)]-2-(4-methoxybenzyl)propanoyl]-*S*-ethyl-L-cysteine (13)**——*N*-Benzoyl-D-phenylalanine (2.83 g, 10.5 mmol) and *N*,*N*′-carbonyldiimidazole (1.95 g, 12 mmol) were stirred in tetrahydrofuran (THF) (40 ml) at −17 °C for 1 h, then **5d** (3.57 g, 10 mmol) in THF (15 ml) was added, and the mixture was allowed to warm gradually to room temperature for 3 h. The reaction mixture was concentrated, the residue was dissolved in EtOAc and water, and the solution was acidified with HCl. The EtOAc layer was washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was chromatographed ($CHCl_3$–MeOH) to give 1.78 g (29%) of **13**. mp 74—88 °C. $[\alpha]_D^{25}$ +9.2° (c=2, EtOH). *Anal.* Calcd for $C_{32}H_{36}N_2O_6S_2 \cdot 1/2H_2O$: C, 62.21; H, 6.04; N, 4.53. Found: C, 62.09; H, 5.99; N, 4.57.

The corresponding *N*-[3-[*N*-benzoyl-D-(alanylthio)]-2-(4-methoxybenzyl)propanoyl)]-*S*-ethyl-L-cysteine (**12**) was similarly synthesized.

12: Amorphous powder. $[\alpha]_D^{25}$ −14.6° (c=2, EtOH). DCHA salt: mp 143—148 °C. *Anal.* Calcd for $C_{26}H_{32}N_2O_6S_2 \cdot C_{12}H_{23}N \cdot 1/3H_2O$: C, 63.39; H, 7.79; N, 5.84. Found: C, 63.40; H, 7.87; N, 5.84.

Biological Methods——The *in vitro* ACE-inhibitory activity was determined according to the procedure described previously.[1]

The *in vivo* ACE-inhibitory activity was examined in male Wistar rats weighing 300—350 g. For at least 7 d prior to the experiments, the rats were fed a standard diet and received tap water *ad libitum*. The animals were anesthetized with urethane (1 g/kg, i.p.). After tracheostomy, polyethylene cannulas were positioned in the femoral vein for drug injection and in the femoral artery for arterial blood pressure measurement monitored with a pressure transducer (Nihon Kohden AP 601G). The blood response to angiotensin I (50 ng/kg) and the depressor response to bradykinin (500 ng/kg) were measured before and after administration of the test compounds.

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Synthesis of 2-Phenylthiazolidine Derivatives as Cardiotonic Agents. II.[1] 2-(Phenylpiperazinoalkoxyphenyl)thiazolidine-3-thiocarboxamides and the Corresponding Carboxamides

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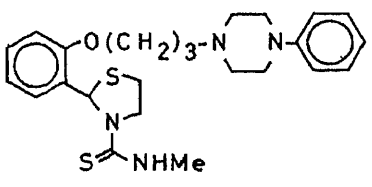
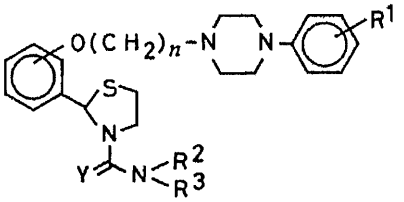
A large number of 2-(phenylpiperazinoalkoxyphenyl)thiazolidine-3-thiocarboxamides and the corresponding carboxamides (II) were synthesized and tested for inotropic activity in anesthetized dogs. Compounds II were prepared from a hydroxybenzaldehyde (III) through the intermediates (IV, V, and X). Structure–activity relationships (SAR) were investigated by varying the structural parameters. Transposition of the piperazinoalkoxyl group to the *meta* or *para* position from the *ortho* position caused a marked fall in activity. Conversion of the thiocarboxamido to a carboxamido group caused a marked increase in activity. This tendency was generally observed in this series of compounds and constitutes a major deviation from the SAR in the simple 2-phenylthiazolidine series. With regard to effects of the length of the aminoalkoxy chain, the ethoxy derivatives were generally more potent than higher analogues. Lengthening of the *N*-alkyl group in the (thio)carboxamido group generally caused a decrease in activity. Among the various derivatives synthesized, II_{15} was found to be approximately one hundred times more potent than amrinone with a long duration of action.

Keywords——2-phenylthiazolidine-3-carboxamide; 2-(phenylpiperazinoalkoxyphenyl)thiazolidine-3-thiocarboxamide; 2-(phenylpiperazinoalkoxyphenyl)thiazolidine-3-carboxamide; positive inotropic activity; structure–activity relationship; cardiotonic agent

The preceding paper[1] of this series described the synthesis and cardiotonic activity of a series of new 2-phenylthiazolidine-3-thiocarboxamides. After examination of the structure–activity relationships (SAR) of numerous derivatives, *N*-methyl-2-(2-(3-(4-phenylpiperazino)-propoxy)phenyl)thiazolidine-3-thiocarboxamide (I) was found to exhibit potent and long-lasting positive inotropic activity in anesthetized dogs. Further exploration of this new lead compound (I) as a cardiotonic agent led to the synthesis of a large number of derivatives represented by general formula (II). This paper describes the synthesis and positive inotropic



I



II

Chart 1

activity of II, and the SAR are discussed in terms of the effect of varying the substituents (R^1, R^2, R^3, and Y), the position of the aminoalkoxyl group, and the length of the alkylene chain (n).

Chemistry

The thiazolidine-3-(thio)carboxamides (II) were synthesized through the sequence of reactions shown in Chart 2. Reaction of hydroxybenzaldehydes (III) with cysteamine followed by treatment with methyl isocyanate (MeNCO) or methyl isothiocyanate (MeNCS) readily gave the phenolic *N*-methylthiazolidine-3-carboxamides or thiocarboxamides (IV). Alkylation of IV with alkylene dihalides or tosyloxyalkyl halides in the presence of potassium carbonate (K_2CO_3) in dimethylformamide (DMF) gave the haloalkoxy derivatives (V) (method A). The reverse procedure (alkylation first followed by thiazolidine formation) also effected the conversion of III to V *via* the aldehydes (VI) (method B). The physical properties of V are summarized in Table I. Treatment of the aldehydes (VI) with ethylene glycol gave the acetals (VII), which in turn were heated with substituted phenylpiperazines[2] and K_2CO_3 in DMF to give, after acidic hydrolysis, the piperazinoalkoxybenzaldehydes (IX) (method C). Alternatively, IX could also be obtained by the condensation of a hydroxybenzaldehyde (III) with the haloalkylpiperazines (VIII) (method D). The physical properties of IX are listed in Table II. Condensation of the halides (V) with various phenylpiperazines (method F) or the reaction of the aldehydes (IX) with cysteamine followed by treatment with MeNCO or MeNCS (method G) gave II (Y=O,S, R^2=H, R^3=Me). Condensation of the phenols (IV, Y=S) with the haloalkylpiperazines (VIII) also gave II (Y=S, R^2=H, R^3=Me) (method E). The physical properties of the *N*-methylthiazolidine-3-carboxamides or thiocarboxamides (II, Y=O,S, R^2=H, R^3=Me) thus obtained are summarized in Table III.

The *N*-alkyl, *N*-phenyl, or *N*-acetylthiazolidine-3-carboxamides or thiocarboxamides (II, Y=O,S, R^2=H, R^3=alkyl, phenyl, or acetyl) were similarly prepared (method G) by the

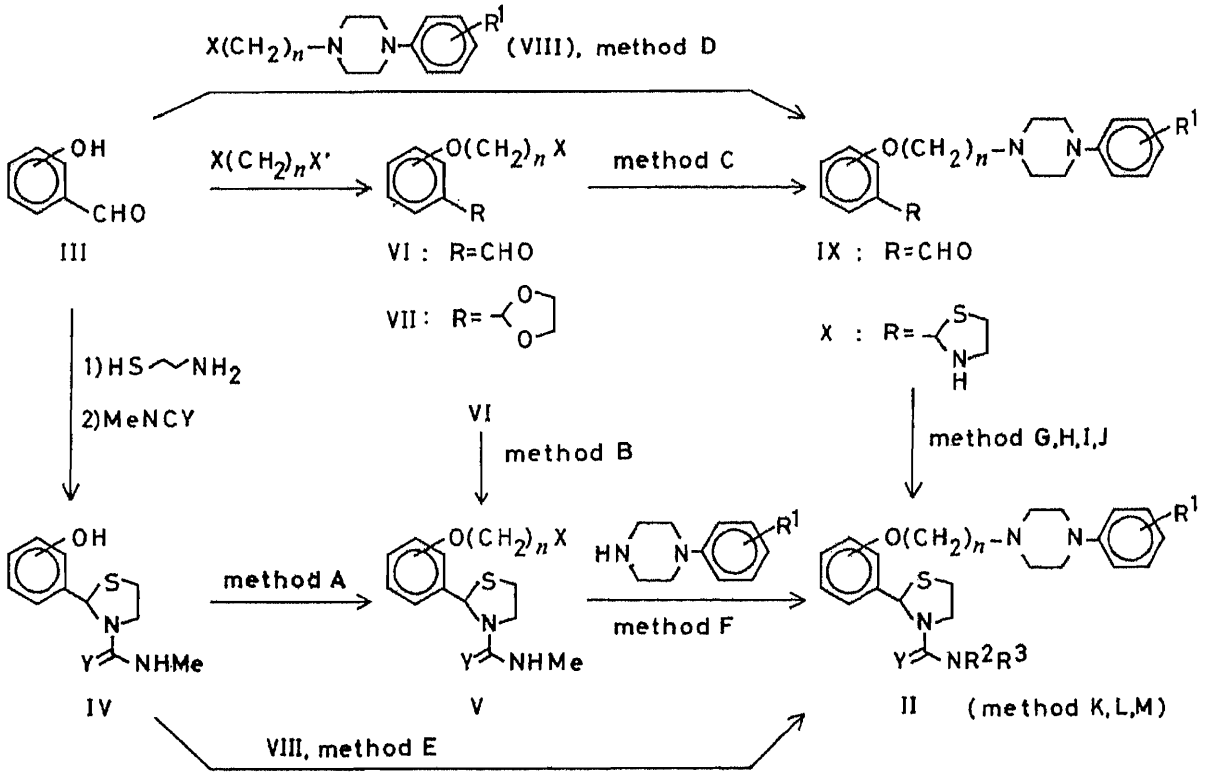


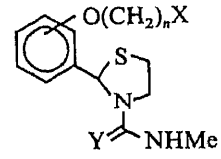
Chart 2

reaction of the intermediate thiazolidines (X) with appropriate isocyanates or isothiocyanates. Alkaline hydrolysis of the *N*-acetylthiocarboxamides (II, Y=S, R^2=H, R^3=Ac) gave the unsubstituted thiocarboxamides (II, Y=S, R^2=R^3=H) (method L). The corresponding carboxamides (II, Y=O, R^2=R^3=H) were obtained by the reaction of X with sodium cyanate (NaOCN) and acetic acid in ethanol (method H). Carbamoylation of X with dimethylcarbamoyl chloride in the presence of K_2CO_3 in DMF gave the *N*,*N*-dimethylcarboxamides (II, Y=O, R^2=R^3=Me) (method I). The reaction of X with thiophosgene followed by treatment with dimethylamine gave the *N*,*N*-dimethylthiocarboxamides (II, Y=S, R^2=R^3=Me) (method J). Acetylation of the *N*-methylthiocarboxamides (II, Y=S, R^2=H, R^3=Me) with acetyl chloride in the presence of sodium hydride in DMF gave the *N*-acetyl[3] derivatives (II, Y=S, R^2=Ac, R^3=Me) (method K). The *N*-acetyl-*N*-methylcarboxamides (II, Y=O, R^2=Ac, R^3=Me) were prepared similarly. Acetylation of the carboxamides (II, Y=O, R^2=R^3=H) with acetyl chloride and triethylamine gave diacetyl compounds,[4] which were hydrolyzed with aqueous sodium hydroxide to give the *N*-acetyl derivatives (II, Y=O, R^2=H, R^3=Ac) (method M). The physical properties of these *N*-substituted thiazolidine-3-carboxamides or thiocarboxamides are listed in Table IV.

Pharmacology and Structure–Activity Relationships

The positive inotropic activity of a series of 2-(phenylpiperazinoalkoxyphenyl)-thiazolidine-3-(thio)carboxamides (II) was determined by measuring the increase in the maximum derivative of left ventricular pressure ($LVdP/dt_{max}$) after i.v. administration to anesthetized dogs by the method reported previously.[5] The results are included in Tables III

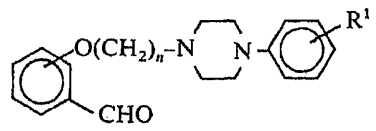
TABLE I. 2-(Haloalkoxyphenyl)-*N*-methylthiazolidine-3-(thio)carboxamides (V)



Compd. No.	Position[a]	*n*	X	Y	Method	Yield (%)	mp (°C) Recrystn. solvent[b]	Formula	Analysis (%) Calcd (Found)		
									C	H	N
a	2	2	Cl	S	B	71	153—154.5 (A)	$C_{13}H_{17}ClN_2OS_2$	49.28 (49.37	5.41 5.36	8.84 8.77)
b	2	2	Cl	O	B	70	166—167 (A)	$C_{13}H_{17}ClN_2O_2S$	51.91 (52.12	5.70 5.65	9.31 9.27)
c	2	3	Cl	S	B	53	118—120 (B-C)	$C_{14}H_{19}ClN_2OS_2$	50.82 (50.92	5.79 5.71	8.47 8.55)
d	2	3	Cl	O	B	63	128—130 (D-E)	$C_{14}H_{19}ClN_2O_2S$	53.41 (53.58	6.08 6.05	8.90 8.93)
e	2	4	Cl	S	B	47	137—143 (A)	$C_{15}H_{21}ClN_2OS_2$	52.23 (52.32	6.14 6.12	8.12 8.14)
f	4	2	Cl	S	A	67	114—116 (A)	$C_{13}H_{17}ClN_2OS_2$	49.28 (49.33	5.41 5.36	8.84 8.87)
g	4	2	Br	O	B	72	96—98.5 (B-C)	$C_{13}H_{17}BrN_2O_2S$	45.23 (45.49	4.96 4.93	8.11 8.20)
h	4	3	Cl	O	B	87	75—78 (B-C)	$C_{14}H_{19}ClN_2O_2S$	53.41 (53.63	6.08 6.12	8.90 9.05)

a) The position of the haloalkoxy group. *b*) A=EtOH, B=AcOEt, C=hexane, D=MeOH, E=Et_2O.

TABLE II. 2-(4-Phenylpiperazinoalkoxy)benzaldehydes (IX)



Compd. No.	Position[a]	n	R^1	Method	Yield (%)	mp (°C) (Recrystn. solvent[b])	^{1}H-NMR δ ($CDCl_3$, J=Hz)
1	2	2	H	C	63	79—82 (F)	2.96—2.84 (6H, m), 3.16—3.30 (4H, m), 4.24 (2H, t, J=5.5), 6.84—7.93 (9H, m), 10.52 (1H, s)
2	2	2	2-Cl	C	35	Oil	2.5—3.4 (10H, m), 4.27 (2H, t, J=6), 6.7—8.1 (8H, m), 10.53 (1H, s)
3	2	2	3-Cl	C	44	Oil	2.6—3.4 (10H, m), 4.25 (2H, t, J=6), 6.60—7.95 (8H, m), 10.49 (1H, s)
4	2	2	4-Cl	C	65	95—105 (E)	2.65—3.26 (10H, m), 4.25 (2H, t, J=6), 6.73—7.92 (8H, m), 10.48 (1H, s)
5	2	2	2-F	C	62	Oil	2.6—3.2 (10H, m), 4.27 (2H, t, J=6), 6.8—7.9 (8H, m), 10.58 (1H, s)
6	2	2	3-F	D	57	76—78 (E-C)	2.6—3.5 (10H, m), 4.30 (2H, t, J=6), 6.35—8.00 (8H, m), 10.52 (1H, s)
7	2	2	4-F	C	67	68—74 (E-C)	2.65—3.30 (10H, m), 4.25 (2H, t, J=6), 6.80—7.95 (8H, m), 10.47 (1H, s)
8	2	2	3-Me	C	38	Oil	2.32 (3H, s), 2.71—3.26 (10H, m), 4.26 (2H, t, J=5.5), 6.66—7.90 (8H, m), 10.52 (1H, s)
9	2	2	4-Me	C	44	Oil	2.26 (3H, s), 2.71—3.21 (10H, m), 4.27 (2H, t, J=5.5), 6.79—7.89 (8H, m), 10.52 (1H, s)
10	2	2	2-OMe	C	57	78—80 (E-C)	2.7—3.2 (10H, m), 3.80 (3H, s), 4.22 (2H, t, J=6), 6.85—7.95 (8H, m), 10.46 (1H, s)
11	2	2	3-OMe	C	51	Oil	2.68—3.26 (10H, m), 3.77 (3H, s), 4.25 (2H, t, J=5.5), 6.34—7.89 (8H, m), 10.51 (1H, s)
12	2	2	4-OMe	C	34	Oil	2.85—3.35 (10H, m), 3.75 (3H, s), 4.39 (2H, t, J=5), 6.88—7.92 (8H, m), 10.46 (1H, s)
13	2	2	4-NO_2	C	45	139—142 (B-C)	2.67—3.03 (6H, m), 3.36—3.52 (4H, m), 4.27 (2H, t, J=5.5), 6.71—8.19 (8H, m), 10.49 (1H, s)
14	2	3	2-Cl	C	54	Oil	1.8—2.4 (2H, m), 2.5—2.9 (6H, m), 3.0—3.3 (4H, m), 4.19 (2H, t, J=6), 6.8—8.0 (8H, m), 10.52 (1H, s)
15	2	3	3-Cl	D	61	86—90 (E-C)	1.85—2.40 (2H, m), 2.5—2.9 (6H, m), 3.1—3.4 (4H, m), 4.15 (2H, t, J=6), 6.65—8.00 (8H, m), 10.46 (1H, s)
16	2	3	4-Cl	C	25	Oil	2.01—2.26 (2H, m), 2.50—2.67 (6H, m), 2.97—3.22 (4H, m), 4.15 (2H, t, J=6), 6.78, 7.13 (2H, each, ABq, J=9), 6.8—7.9 (4H, m), 10.44 (1H, s)
17	2	3	2-F	C	68	81—84 (F)	1.95—2.40 (2H, m), 2.55—2.90 (6H, m), 3.05—3.35 (4H, m), 4.18 (2H, t, J=6), 6.90—8.00 (8H, m), 10.50 (1H, s)
18	2	3	3-F	D	63	65—79 (E-C)	1.8—2.4 (2H, m), 2.5—2.8 (6H, m), 3.1—3.35 (4H, m), 4.17 (2H, t, J=6), 6.8—7.95 (8H, m), 10.52 (1H, s)
19	2	3	4-F	C	80	54—57 (E-C)	1.9—2.3 (2H, m), 2.52—2.79 (6H, m), 3.00—3.24 (4H, m), 4.16 (2H, t, J=6), 6.84—7.93 (8H, m), 10.49 (1H, s)
20	2	3	3-Me	D	59	53—54 (E-C)	1.8—2.3 (2H, m), 2.31 (3H, s), 2.45—2.90 (6H, m), 3.15—3.40 (4H, m), 4.16 (2H, t, J=6), 6.55—7.95 (8H, m), 10.47 (1H, s)

TABLE II. (continued)

Compd. No.	Position[a]	n	R^1	Method	Yield (%)	mp (°C) (Recrystn. solvent[b])	^{1}H-NMR δ ($CDCl_3$, J=Hz)
21	2	3	4-Me	C	89	46—51 (E–C)	2.00—2.25 (2H, m), 2.25 (3H, s), 2.52—2.68 (6H, m), 3.06—3.22 (4H, m), 4.15 (2H, t, J=6), 6.70—7.90 (8H, m), 10.45 (1H, s)
22	2	3	2-OMe	C	67	104—108 (B–C)	1.9—2.3 (2H, m), 2.45—2.90 (6H, m), 2.95—3.30 (4H, m), 3.85 (3H, s), 4.18 (2H, t, J=6), 6.80—7.95 (8H, m), 10.49 (1H, s)
23	2	3	3-OMe	C	46	Oil	1.90—2.20 (2H, m), 2.52—3.26 (10H, m), 3.78 (3H, s), 4.16 (2H, t, J=6), 6.34—7.88 (8H, m), 10.51 (1H, s)
24	2	3	4-OMe	C	52	58—62 (E–C)	1.97—2.28 (2H, m), 2.55—2.71 (6H, m), 2.99—3.19 (4H, m), 3.76 (3H, s), 4.18 (2H, t, J=6), 6.87—7.95 (8H, m), 10.53 (1H, s)
25	2	3	4-NO_2	C	62	121—123.5 (B–C)	1.85—2.35 (2H, m), 2.52—2.75 (6H, m), 3.34—3.51 (4H, m), 4.18 (2H, t, J=6), 6.70—8.16 (8H, m), 10.49 (1H, s)
26	3	3	H	D	30	Oil	1.94—2.25 (2H, m), 2.63—2.85 (6H, m), 3.27—3.43 (4H, m), 4.24 (2H, t, J=6), 6.85—7.50 (9H, m), 10.10 (1H, s)
27	4	3	H	D	30	Oil	1.90—2.27 (2H, m), 2.50—2.94 (6H, m), 3.14—3.30 (4H, m), 4.14 (2H, t, J=6), 6.85—7.92 (9H, m), 9.87 (1H, s)
28	2	5	H	C	61	Oil	1.48—1.97 (6H, m), 2.37—2.67 (6H, m), 3.17—3.27 (4H, m), 4.10 (2H, t, J=6), 6.84—7.88 (9H, m), 10.52 (1H, s)

a) The position of the phenylpiperazinoalkoxy group. *b*) F=isopropyl ether and see also footnote *b*) in Table I.

and IV together with comparative data for amrinone.

The effects of varying the position of the piperazinoalkoxyl group in the benzene ring of I were examined first. Transposition of this group to the *meta* position (II_{41}) from the *ortho* position (I) caused a marked fall in activity. On the other hand, the *para* thiocarboxamides ($II_{42,44}$) retained a considerable degree of activity. In the carboxamide series, the *ortho* isomers ($II_{15,36}$) exhibited highly potent activity, whereas the corresponding *para* isomers ($II_{43,45}$) rather decreased the contractile force. The favorable effect of the *ortho* substitution is consistent with our earlier finding in the simple 2-phenyl derivatives.[1)]

Conversion of the thiocarboxamido group of I to a carboxamide group caused a marked increase in activity. The carboxamide (II_{36}) was approximately ten times as potent as I. An about thirtyfold increase in activity was also occasioned by the conversion of the thiocarboxamide (II_1) to the corresponding carboxamide (II_{15}). This tendency was generally observed in this series of compounds and constitutes a major deviation from the SAR in the 2-methoxyphenyl series, where the conversion of the thiocarboxamido group to the carboxamido group caused a marked decrease in activity.[1)]

With regard to the effect of the length of the aminoalkoxy chain on activity, the ethoxy derivatives were generally more potent than the propoxy analogues. This tendency was more pronounced in the carboxamide series (*e.g.* II_{15} *vs.* II_{36} and II_{17} *vs.* II_{37}) than in the thiocarboxamide series (*e.g.* II_1 *vs.* I and II_5 *vs.* II_{26}). Further lengthening of the alkylene chain caused a decrease in activity as shown by the pentyloxythiocarboxamide (II_{40}).

The effects of substitution on the benzene ring of the piperazine moiety were extensively examined in the thiocarboxamide series (II_{1-14} and II_{23-35}). Although no clear SAR can be

deduced, introduction of a fluoro group (II_{5-7} and II_{26-28}) tends to have a favorable effect. In the carboxamide series, the unsubstituted phenyl (II_{15}) and 2-fluorophenyl (II_{17}) derivatives exhibited the most potent activity. This was followed by the 3-fluoro (II_{18}), 4-fluoro (II_{19}), and 2-methyl (II_{20}) derivatives.

The effect of modifying the *N*-substituent in the (thio)carboxamido group was examined in a series of the *ortho* piperazinoethoxy derivatives listed in Table IV. The unsubstituted ($II_{46,49,57}$) and *N*-acetyl ($II_{47,52,55,61}$) derivatives exhibited potent activity, comparable to the corresponding *N*-methyl derivatives. This also constitutes a deviation from the SAR in the 2-methoxyphenyl series.[1] The extraordinarily long-lasting positive inotropic action with a slow onset induced by the *N*-acetate (II_{61}) may arise, at least in part, from its metabolic transformation. Lengthening of the *N*-alkyl group generally caused a decrease in activity ($II_{50-51,58,59}$). *N*-Dimethyl substitution in the carboxamido group caused a decrease in activity ($II_{48,54}$), while the corresponding thiocarboxamides ($II_{56,63}$) exhibited activity comparable to the corresponding monomethyl derivatives. Thus, there are some discrepancies in the SAR between the carboxamide and thiocarboxamide series and also between the 2-methoxyphenyl[1] and the piperazinoalkoxyphenyl series.

As a consequence of the above SAR, *N*-methyl-2-(2-(2-(4-phenylpiperazino)-ethoxy)phenyl)thiazolidine-3-carboxamide (II_{15}), which was approximately one hundred times more potent than amrinone, was selected for further study. In the isolated guinea pig heart, its minimum effective dose (MED) to cause an increase in contractile force[6] was 3 μg/heart (MED for amrinone = 10 μg/heart). After intraduodenal administration of 0.1 mg/kg to anesthetized dogs, II_{15} produced a 45% increase in $LVdP/dt_{max}$ with a 20% increase in heart rate; the positive inotropic action persisted for more than 2 h without affecting mean blood pressure. Oral administration of 0.3 mg/kg of II_{15}[7] to conscious instrumented dogs also produced potent positive inotropic action (a 26% increase in $LVdP/dt_{max}$) lasting for 5 h. Compound (II_{15}) had low toxicity in mice ($LD_{50} > 1000$ mg/kg *p.o.*).

Further studies on the synthesis and SAR of 2-phenylthiazolidine-3-(thio)carboxamides as new cardiotonic agents are in progress.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi IR-215 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz on a JEOL PMX-60 spectrometer with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument.

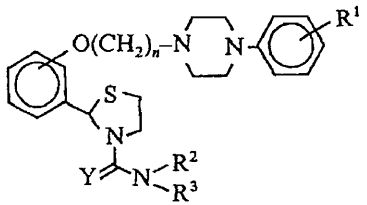
2-(2-Hydroxyphenyl)-*N*-methylthiazolidine-3-carboxamide (IVa, *o*-Isomer, Y = O)——This compound was prepared according to the reported procedure[1] from salicylaldehyde, cysteamine, and methyl isocyanate in 75% yield. mp 186—188 °C (dec.) (EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3430, 3350, 3280, 1620. ^{1}H-NMR ($CDCl_3$) δ: 2.70 (3H, d, J = 4.5 Hz), 2.90—3.25 (2H, m), 3.45—4.40 (2H, m), 5.60 (1H, br), 6.32 (1H, s), 6.62—7.35 (4H, m), 9.59 (1H, s). MS *m/z*: 238 (M^+). *Anal.* Calcd for $C_{11}H_{14}N_2O_2S$: C, 55.44; H, 5.92; N, 11.76; S, 13.45. Found: C, 55.43; H, 5.90; N, 11.80; S, 13.32. The following compounds were prepared in a similar manner.

2-(4-Hydroxyphenyl)-*N*-methylthiazolidine-3-carboxamide (IVb, *p*-Isomer, Y = O)——68% yeild. mp 218—220 °C (EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3410, 3310, 1620. ^{1}H-NMR ($CDCl_3$) δ: 2.68 (3H, d, J = 4.5 Hz), 2.90—3.13 (2H, m), 3.55—4.35 (2H, m), 5.30 (1H, br), 6.08 (1H, s), 6.76, 7.11 (2H each, ABq, J = 9 Hz), 8.94 (1H, s). MS *m/z*: 238 (M^+). *Anal.* Calcd for $C_{11}H_{14}N_2O_2S$: C, 55.44; H, 5.92; N, 11.76; S, 13.45. Found: C, 55.28; H, 5.84; N, 11.69; S, 13.41.

2-(4-Hydroxyphenyl)-*N*-methylthiazolidine-3-thiocarboxamide (IVc, *p*-Isomer, Y = S)——58% yield. mp 152—153 °C (EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3280, 1610. MS *m/z*: 254 (M^+). *Anal.* Calcd for $C_{11}H_{14}N_2OS_2$: C, 51.94; H, 5.55; N, 11.01. Found: C, 52.09; H, 5.52; N, 11.19.

2-(4-(2-Chloroethoxy)phenyl)-*N*-methylthiazolidine-3-thiocarboxamide (Vf, *p*-Isomer, *n* = 2, X = Cl, Y = S)——Method A: A mixture of IVc (*p*-isomer, Y = S, 5.0 g, 19.6 mmol), 1-chloro-2-tosyloxyethane (4.6 g, 19.6 mmol), and K_2CO_3 (4.0 g, 29.0 mmol) in DMF (20 ml) was stirred at 50 °C overnight. The mixture was diluted with water and

TABLE III. 2-(4-Phenylpiperazinoalkoxyphenyl)thiazolidine-3-(thio)carboxamides (II, $R^2=H$, $R^3=CH_3$)



Compd. No.	Position[a]	n	Y	R^1	Method	Yield (%)	Salt[b]	mp (°C) (Recrystn. solvent[c])	Analysis (%)[e] Calcd (Found)			Myocardial contractility Anesthetized dog[f]		
									C	H	N	Dose (mg/kg) i.v.	LVdP/dt_{max} (Δ%)	Duration (min)
1	2	2	S	H	G	72	—	124—126 (B–C)	$C_{27}H_{34}N_4OS_2$			0.1	27	20
							fum	166—168[d] (A–G)	58.04 (58.02	6.03 6.03	9.92 9.92)			
2	2	2	S	2-Cl	G	79	—	145—151 (E–C)	$C_{27}H_{33}ClN_4O_5S_2$			0.3	22	14
							fum	180—181[d] (D–G–C)	54.67 (54.76	5.61 5.66	9.45 9.49)			
3	2	2	S	3-Cl	G	58	—	147—148 (D–E)	$C_{27}H_{33}ClN_4O_5S_2$			0.1	17	>30
							fum	151—153[d] (D–E)	54.67 (54.47	5.61 5.67	9.45 9.50)			
4	2	2	S	4-Cl	G	52	—	130—133 (E)	$C_{27}H_{33}ClN_4O_5S_2$			0.3	21	30
							fum	155—160[d] (G–E)	54.67 (54.40	5.61 5.61	9.45 9.55)			
5	2	2	S	2-F	G	70	—	143—145 (B–C)	$C_{27}H_{33}FN_4O_5S_2$			0.1	37	>30
							fum	178—179[d] (D–E)	56.23 (56.06	5.77 5.72	9.72 9.72)			

6	2	2	S	3-F	G	92	—	132—134 (B–C)	$C_{27}H_{33}FN_4O_5S_2$			0.1	38	> 30
							fum	164[d] (D–G)	56.23 (55.98	5.77 5.75	9.72 9.63)			
7	2	2	S	4-F	G	65	—	132—133 (B–C)	$C_{27}H_{33}FN_4O_5S_2$			0.1	20	22
							fum	163—164[d] (D–G–C)	56.23 (56.11	5.77 5.72	9.72 9.68)			
8	2	2	S	2-CH_3	G	68	—	171—174 (B–A)	$C_{28}H_{36}N_4O_5S_2$			0.1	41	60
							fum	186—188[d] (G)	58.72 (58.58	6.34 6.28	9.78 9.81)			
9	2	2	S	3-CH_3	G	41	—	132—136 (A)	$C_{28}H_{36}N_4O_5S_2 \cdot 1/2\,H_2O$			0.1	23	27
							fum	157—160[d] (G)	57.81 (57.64	6.41 6.20	9.63 9.63)			
10	2	2	S	4-CH_3	G	71	—	157—160 (A)	$C_{26}H_{34}N_4O_5S_2 \cdot 1/2\,H_2O$			0.3	27	42
							1/2 fum	170—171.5[d] (A)	59.63 (60.01	6.74 6.71	10.70 10.55)			
11	2	2	S	2-OCH_3	G	86	—	161—162.5 (E)	$C_{28}O_{36}N_4O_6S_2 \cdot H_2O$			0.3	20	8.5
							fum	181—183[d] (D–G–C)	55.43 (55.38	6.31 6.13	9.23 9.27)			
12	2	2	S	3-OCH_3	G	50	—	135—138 (A–F)	$C_{28}H_{36}N_4O_6S_2$			0.1	27	40
							fum	155—159[d] (A–F)	57.12 (57.24	6.16 6.24	9.52 9.61)			
13	2	2	S	4-OCH_3	G	55	—	135—137 (B–C)	$C_{28}H_{36}N_4O_6S_2$			0.3	30	65
							fum	155—160[d] (A)	57.12 (57.37	6.16 6.36	9.52 9.80)			
14	2	2	S	4-NO_2	G	80	—	116—119 (H–B–C)	$C_{23}H_{30}ClN_5O_3S_2$			1	14	13
							HCl	218—220[d] (I–B)	52.71 (52.54	5.77 5.91	13.36 13.55)			

TABLE III. (continued)

Compd. No.	Position[a]	n	Y	R^1	Method	Yield (%)	Salt[b]	mp (°C) (Recrystn. solvent[c])	Analysis (%)[e] Calcd (Found)			Myocardial contractility Anesthetized dog[f]		
									C	H	N	Dose (mg/kg) i.v.	LVdP/dt_{max} (Δ%)	Duration (min)
15	2	2	O	H	F	58	—	127—129 (B–C)	$C_{25}H_{32}N_4O_6S \cdot 1/2\,H_2O$			0.003	30	30
							ox	152—153.5[d] (G)	57.13 (57.08	6.33 6.12	10.66 10.76)			
16	2	2	O	3-Cl	F	66	—	119—121 (B–E)	$C_{25}H_{31}ClN_4O_6S$			0.03	20	10
							ox	138—139[d] (A–E)	54.49 (54.46	5.67 5.67	10.17 10.20)			
17	2	2	O	2-F	F	90	—	Oil	$C_{25}H_{31}FN_4O_6S$			0.003	20	30
							ox	175—176[d] (G)	56.17 (55.95	5.84 5.80	10.48 10.60)			
18	2	2	O	3-F	G	90	—	156—157 (G–C)	$C_{25}H_{31}FN_4O_6S$			0.01	40	35
							ox	173—175[d] (G)	56.17 (56.11	5.84 5.81	10.48 10.40)			
19	2	2	O	4-F	F	77	—	134—136 (B–C)	$C_{25}H_{31}FN_4O_6S$			0.01	25	20
							ox	120—123[d] (G)	56.17 (56.04	5.81 5.78	10.48 10.52)			
20	2	2	O	2-CH_3	F	73	—	162—163 (H–E)	$C_{26}H_{34}N_4O_6S$			0.01	30	>30
							ox	183—184[d] (A)	58.85 (59.09	6.46 6.47	10.56 10.68)			
21	2	2	O	3-CH_3	F	71	—	112—114 (B–E)	$C_{26}H_{34}N_4O_6S$			0.1	30	10
							ox	153—154[d] (A–E)	58.85 (58.98	6.46 6.56	10.56 10.73)			

22	2	2	O	3-OCH_3	F	78	—	123—124 (H–E)	$C_{26}H_{34}N_4O_7S$			0.1	45	> 30
							ox	151—152[d] (A–E)	57.13 (57.32	6.27 6.30	10.25 10.33)			
23	2	3	S	2-Cl	G	86	—	111—116 (E–C)	$C_{28}H_{35}ClN_4O_5S_2$			0.3	35	50
							fum	149—151.5[d] (G–C)	55.39 (55.34	5.81 5.87	9.23 9.08)			
24	2	3	S	3-Cl	E	52	—	151—153.5 (B–C)	$C_{28}H_{35}ClN_4O_5S_2$			0.3	22	21
							fum	143.5—145[d] (G–C)	55.39 (55.05	5.81 5.82	9.23 9.15)			
25	2	3	S	4-Cl	G	65	—	126—130 (B–C)	$C_{28}H_{35}ClN_4O_5S_2$			0.3	29	25
							fum	133—138 (A–E)	55.39 (55.29	5.81 5.77	9.23 9.17)			
26	2	3	S	2-F	G	71	—	116—118 (B–C–E)	$C_{28}H_{35}FN_4O_5S_2$			0.1	20	34
							fum	138—140[d] (G–C)	56.93 (56.74	5.97 5.94	9.48 9.56)			
27	2	3	S	3-F	G	85	—	133—134 (A–J)	$C_{28}H_{35}FN_4O_5S_2$			0.1	30	> 50
							fum	147—148[d] (G–C)	56.93 (56.83	5.97 6.02	9.48 9.37)			
28	2	3	S	4-F	G	78	—	113—116 (B–C)	$C_{28}H_{35}FN_4O_5S_2$			0.3	20	17
							fum	115—118[d] (A–E–G)	56.93 (56.78	5.97 5.93	9.48 9.24)			
29	2	3	S	2-CH_3	F	91	—	110—112 (E–C)	$C_{29}H_{38}N_4O_5S_2$			0.3	33	> 40
							fum	162—164[d] (G–C)	59.36 (59.36	6.53 6.50	9.55 9.54)			
30	2	3	S	3-CH_3	G	91	—	140—141.5 (A–J)	$C_{29}H_{38}N_4O_5S_2$			0.3	24	35
							fum	140—142 (G–C)	59.36 (59.19	6.53 6.58	9.55 9.37)			

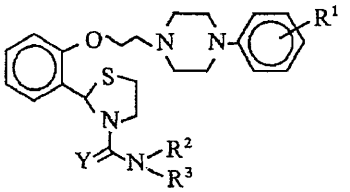
TABLE III. (continued)

Compd. No.	Position[a]	n	Y	R^1	Method	Yield (%)	Salt[b]	mp (°C) (Recrystn. solvent[c])	Analysis (%)[e] Calcd (Found)			Myocardial contractility Anesthetized dog[f]		
									C	H	N	Dose (mg/kg) i.v.	LV$dP/dt_{max}$ (Δ%)	Duration (min)
31	2	3	S	4-CH_3	G	84	—	163—165 (B–C)	$C_{29}H_{38}N_4O_5S_2$			0.3	26	34
							fum	135—138[d] (A–E–G)	59.36 (59.28	6.53 6.59	9.55 9.30)			
32	2	3	S	2-OCH_3	G	69	—	110—115 (E–C)	$C_{29}H_{38}N_4O_6S_2$			0.3	34	25
							fum	159—161 (G–C)	57.78 (57.67	6.35 6.44	9.30 9.07)			
33	2	3	S	3-OCH_3	G	63	—	149—154 (B–C)	$C_{27}H_{36}N_4O_6S_2$			0.3	30	24
							ox	154—157[d] (D–E)	56.23 (56.26	6.29 6.30	9.72 9.63)			
34	2	3	S	4-OCH_3	G	83	—	158—161 (B–C)	$C_{29}H_{38}N_4O_6S_2$			0.3	28	20
							fum	129—132 (A–G)	57.78 (57.53	6.35 6.44	9.30 8.99)			
35	2	3	S	4-NO_2	G	77	—	191—193 (H–B–C)	$C_{26}H_{33}N_5O_7S_2$			0.3	25	42
							ox	197—199[d] (D–E)	52.78 (52.48	5.62 5.56	11.84 12.01)			
36	2	3	O	H	F	66	—	85.5—90 (B–C–E)	$C_{26}H_{34}N_4O_6S$			0.03	26	21
							ox	118—130[d] (A–E)	58.85 (58.75	6.46 6.51	10.56 10.54)			
37	2	3	O	2-F	F	58	—	121—129 (K–B–C)	$C_{26}H_{33}FN_4O_6S$			0.3	20	30
							ox	126—134[d] (A–E)	56.92 (56.87	6.06 6.09	10.21 10.27)			

38	2	3	O	3-F	F	68	—	103—105 (B–C)	$C_{26}H_{33}FN_4O_6S$			0.1	25	30
							ox	150—151.5[d] (A–E–G)	56.92 (56.85	6.06 6.10	10.21 10.21)			
39	2	4	S	H	F	40	—	105—110 (K)	$C_{29}H_{38}N_4O_5S_2$			0.3	34	40
							fum	145—147 (G–C)	59.36 (59.56	6.53 6.49	9.55 9.44)			
40	2	5	S	H	G	34	—	121.5—123.5 (K–F)	$C_{28}H_{38}N_4O_5S_2$			1	−8	
							ox	173—175[d] (A–D–E)	58.51 (58.31	6.66 6.59	9.75 9.73)			
41	3	3	S	H	G	72	—	Oil	$C_{28}H_{36}N_4O_5S_2$			1	5	8
							fum	144—148[d] (A–E–G)	58.72 (58.81	6.34 6.60	9.78 9.64)			
42	4	2	S	H	F	66	—	Oil	$C_{23}H_{33}Cl_3N_4OS_2$			0.3	24	20
							3HCl	164—167[d] (A–E–G)	50.04 (50.15	6.06 6.13	10.15 10.17)			
43	4	2	O	H	F	79	—	85—91 (B–C)	$C_{25}H_{32}N_4O_6S$			1	−8	
							ox	125—130[d] (G)	58.12 (57.98	6.24 6.20	10.85 10.63)			
44	4	3	S	H	G	69	—	146—149 (K)	$C_{26}H_{34}N_4O_5S_2$			0.3	27	65
							ox	115—120 (D–E)	57.12 (56.90	6.27 6.22	10.25 9.99)			
45	4	3	O	H	F	70	—	129—130 (B–C)	$C_{26}H_{34}N_4O_6S$			0.03	−15	
							ox	122—127[d] (G)	58.85 (58.65	6.46 6.41	10.56 10.44)			
I[g]	2	3	S	H								0.3	68	60
Amrinone												0.3	25	25

a) The position of the phenylpiperazinoalkoxy group. *b*) fum = fumarate, ox = oxalate. *c*) G = acetone, H = chloroform, I = dimethylformamide, J = H_2O, K = isopropanol. See also footnote *b* in Tables I and II. *d*) With decomposition. *e*) Elemental analysis for the salt. *f*) Myocardial contractility was examined with the salts. For methodology, see reference 5. *g*) See reference 1.

TABLE IV. 2-(2-(2-(4-Phenylpiperazino)ethoxy)phenyl)thiazolidine-3-(thio)carboxamides (II, *o*-Isomer, $n=2$)



Compd. No.	Y	R^1	R^2	R^3	Method	Yield (%)	Salt[a]	mp (°C) (Recrystn. solvent[b])	Analysis (%)[d] Calcd (Found)			Myocardial contractility Anesthetized dog[e]		
									C	H	N	Dose (mg/kg) i.v.	LV$dP/dt_{max}$ (Δ%)	Duration (min)
46	O	H	H	H	H	82	—	137—139 (B)	$C_{24}H_{30}N_4O_6S$			0.003	34	45
							ox	146—150.5[c] (D–G)	57.35 (57.64	6.06 6.37	11.15 10.95)			
47	O	H	H	$COCH_3$	M	46	—	137—139 (B–C)	$C_{25}H_{31}N_4O_4S \cdot 1/2\,H_2O$			0.003	28	20
							1/2 ox	164—168[c] (G)	59.03 (58.75	6.34 6.02	11.02 10.72)			
48	O	H	CH_3	CH_3	I	58	—	100—102 (F)	$C_{26}H_{34}N_4O_6S$			0.1	21	25
							ox	167—168.5[c] (G)	58.85 (58.83	6.46 6.45	10.56 10.43)			
49	O	3-F	H	H	H	88	—	142—143 (B–E)	$C_{24}H_{29}FN_4O_6S$			0.01	38	20
							ox	104—105[c] (D–E)	55.37 (55.40	5.62 5.60	10.76 10.64)			
50	O	3-F	H	C_2H_5	G	89	—	116—118 (B–C)	$C_{26}H_{33}FN_4O_6S$			0.03	22	23
							ox	197—198[c] (J–G–E)	56.92 (57.08	6.06 6.06	10.21 10.28)			

51	O	3-F	H	C_4H_9	G	71	—	93—95.5 (E–F–C)	$C_{30}H_{39}FN_4O_6S$			0.1	28	3
							fum	165—167[c] (A–F)	59.78 (59.64	6.52 6.52	9.30 9.33)			
52	O	3-F	H	$COCH_3$	M	50	—	142—143 (B–E)	$C_{26}H_{31}FN_4O_7S$			0.01	26	40
							ox	165—166[c] (A)	55.50 (55.60	5.55 5.63	9.96 10.01)			
53	O	3-F	$COCH_3$	CH_3	K	29	—	Oil	$C_{27}H_{33}FN_4O_7S$			0.03	39	35
							ox	110—117[c] (D–G–C)	56.24 (56.45	5.77 5.79	9.72 9.58)			
54	O	3-F	CH_3	CH_3	I	50	—	90.5—93 (F)	$C_{26}H_{33}FN_4O_6S$			0.03	74	33
							ox	171—172.5[c] (A–G–E)	56.92 (56.96	6.06 6.09	10.21 10.19)			
55	S	H	H	$COCH_3$	G	56	—	139—140.5 (B–C)	$C_{26}H_{32}N_4O_6S_2$			0.1	22	25
							ox	156—157[c] (G)	55.69 (55.72	5.75 5.83	9.99 9.77)			
56	S	H	CH_3	CH_3	J	46	—	Oil	$C_{26}H_{34}N_4O_5S_2$			0.1	32	30
							ox	90—100 (G)	57.12 (57.14	6.27 6.30	10.25 10.13)			
57	S	3-F	H	H	L	90	—	136—137 (E)	$C_{24}H_{29}FN_4O_3S$			0.03	44	23
							1/2 fum	164—165[c] (A–E)	57.12 (57.24	5.79 5.84	11.10 11.06)			
58	S	3-F	H	C_2H_5	G	99	—	Oil	$C_{26}H_{33}FN_4O_5S_2$			0.3	30	25
							ox	148—151[c] (G)	55.30 (55.01	5.89 6.08	9.92 9.65)			
59	S	3-F	H	C_4H_9	G	94	—	Oil	$C_{30}H_{39}FN_4O_5S_2 \cdot 1/2\,H_2O$			0.3	27	32
							fum	168—170.5[c] (G–C)	57.40 (57.65	6.42 6.25	8.92 8.79)			

TABLE IV. (continued)

Compd. No.	Y	R^1	R^2	R^3	Method	Yield (%)	Salt[a]	mp (°C) (Recrystn. solvent[b])	Analysis (%)[d] Calcd (Found)			Myocardial contractility Anesthetized dog[e]		
									C	H	N	Dose (mg/kg) i.v.	LV$dP/dt_{max}$ (Δ%)	Duration (min)
60	S	3-F	H	C_6H_5	G	62	—	124—126 (B–C)	$C_{32}H_{35}FN_4O_5S_2$			0.3	29	7
							fum	161.5—164[c] (A–D)	60.17 (59.94	5.52 5.42	8.77 8.70)			
61	S	3-F	H	$COCH_3$	G	49	—	151—152 (A–E)	$C_{26}H_{31}FN_4O_6S_2$			0.1	50	190
							ox	121—122[c] (A–E)	53.96 (53.71	5.40 5.47	9.68 9.66)			
62	S	3-F	$COCH_3$	CH_3	K	77	—	Oil	$C_{27}H_{33}FN_4O_6S_2$			0.1	30	20
							ox	106—109[c] (G–E)	54.71 (54.61	5.61 5.63	9.45 9.47)			
63	S	3-F	CH_3	CH_3	J	60	—	Oil	$C_{26}H_{33}FN_4O_5S_2$			0.1	36	20
							ox	79—84 (G)	55.30 (55.59	5.89 5.82	9.92 10.00)			

a—*e*) See footnote *b*—*f*, respectively, in Table III.

extracted with AcOEt. The extracts were washed successively with 10% aq. NaOH and water, and evaporated. The residue was purified by silica gel chromatography with AcOEt–$CHCl_3$ (1 : 4) and recrystallized from EtOH to give 3.95 g of Vf as colorless prisms. IR ν_{max}^{Nujol} cm^{-1}: 3325. ^{1}H-NMR ($CDCl_3$) δ: 3.02 (3H, d, J=4 Hz), 2.98—3.20 (2H, m), 3.79 (2H, t, J=5 Hz), 4.22 (2H, t, J=5 Hz), 4.13—4.74 (2H, m), 5.44 (1H, br), 6.21 (1H, s), 6.88, 7.22 (2H each, ABq, J=9 Hz), MS m/z: 318, 316 (M^+).

2-(2-Chloroethoxy)benzaldehyde (VIa, *o*-Isomer, n=2, X=Cl)——A mixture of salicylaldehyde (40 g, 0.328 mol), 1-chloro-2-tosyloxyethane (84 g, 0.358 mol), and K_2CO_3 (50 g, 0.362 mol) in DMF (270 ml) was stirred at room temperature for 68 h. The mixture was poured into water and the liberated oil was extracted with Et_2O. The extracts were washed with 10% aq. NaOH, H_2O, dried, and evaporated. The residue was distilled under reduced pressure to give 51.8 g (86%) of VIa[8] as an oil. bp 116—118 °C (0.2 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 3.84 (2H, t, J=5.5 Hz), 4.33 (2H, t, J=5.5 Hz), 6.86—7.87 (4H, m), 10.47 (1H, s). The following compounds were prepared in a similar manner.

2-(3-Chloropropoxy)benzaldehyde (VIb, *o*-Isomer, n=3, X=Cl)——85% yield. bp 125—129 °C (0.3 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 2.30 (2H, m), 3.76 (2H, t, J=6 Hz), 4.24 (2H, t, J=6 Hz), 6.80—7.89 (4H, m), 10.44 (1H, s).

2-(4-Chlorobutoxy)benzaldehyde (VIc, *o*-Isomer, n=4, X=Cl)——95% yield. bp 142—144 °C (0.35 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.93—2.11 (4H, m), 3.63 (2H, distorted t, J=6 Hz), 4.12 (2H, distorted t, J=6 Hz), 6.88—7.89 (4H, m), 10.47 (1H, s).

2-(5-Bromopentyloxy)benzaldehyde (VId, *o*-Isomer, n=5, X=Br)——72% yield. bp 148—157 °C (0.35 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.45—2.10 (6H, m), 3.44 (2H, t, J=6.5 Hz), 4.10 (2H, t, J=5.8 Hz), 6.91—7.88 (4H, m), 10.51 (1H, s).

4-(2-Bromoethoxy)benzaldehyde (VIe, *p*-Isomer, n=2, X=Br)——21% yield. bp 119—132 °C (0.4 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 3.63 (2H, t, J=6 Hz), 4.35 (2H, t, J=6 Hz), 6.95, 7.77 (2H each, ABq, J=9 Hz), 9.81 (1H, s).

4-(3-Chloropropoxy)benzaldehyde (VIf, *p*-Isomer, n=3, X=Cl)——77% yield. bp 130—140 °C (0.35 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 2.26 (2H, m), 3.75 (2H, t, J=6 Hz), 4.20 (2H, t, J=6 Hz), 7.00, 7.82 (2H, each ABq, J=9 Hz), 9.86 (1H, s).

2-(2-(2-Chloroethoxy)phenyl)-*N*-methylthiazolidine-3-thiocarboxamide (Va, *o*-Isomer, n=2, X=Cl, Y=S)——Method B: This compound was prepared from VIa, cysteamine, and methyl isothiocyanate by the method of the preceding paper.[1] IR ν_{max}^{Nujol} cm^{-1}: 3325. ^{1}H-NMR ($CDCl_3$) δ: 3.02 (3H, d, J=4.5 Hz), 3.10 (2H, t, J=5 Hz), 3.80—4.01 (2H, m), 4.12—4.85 (4H, m), 5.50 (1H, br), 6.41 (1H, s), 6.85—7.47 (4H, m). Compounds Vb—e, g, h were prepared in a similar manner and their physical properties are summarized in Table I.

2-(2-Chloroethoxy)benzaldehyde Ethylene Acetal (VIIa, *o*-Isomer, n=2, X=Cl)——A solution of VIa (46.5 g, 0.25 mol), ethylene glycol (33.2 g, 0.53 mol), and 85% phosphoric acid (0.5 ml) in benzene (500 ml) was refluxed for 18 h with a Dean-Stark water separator. The reaction mixture was cooled to room temperature and washed with aq. $NaHCO_3$. The organic layer was washed with water, dried, and evaporated. The residue was distilled under reduced pressure to afford 56 g (97%) of VIIa as an oil. bp 130—135 °C (0.3 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 3.71—4.37 (8H, m), 6.18 (1H, s), 6.80—7.60 (4H, m). The following compounds were prepared in a similar manner.

2-(3-Chloropropoxy)benzaldehyde Ethylene Acetal (VIIb, *o*-Isomer, n=3, X=Cl)——94% yield. bp 136—137 °C (0.2 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 2.32 (2H, m), 3.81 (2H, t, J=6 Hz), 4.13 (4H, m), 4.22 (2H, t, J=6 Hz), 6.20 (1H, s), 6.79—7.63 (4H, m).

2-(4-Chlorobutoxy)benzaldehyde Ethylene Acetal (VIIc, *o*-Isomer, n=4, X=Cl)——90% yield. bp 165—167 °C (0.4 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.65—2.25 (4H, m), 3.48—3.80 (2H, m), 3.90—4.20 (6H, m), 6.15 (1H, s), 6.80—7.59 (4H, m).

2-(5-Bromopentyloxy)benzaldehyde Ethylene Acetal (VIId, *o*-Isomer, n=5, X=Br)——88% yield. bp 160—165 °C (0.35 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.72—2.12 (6H, m), 3.34 (2H, t, J=6 Hz), 3.84—4.17 (6H, m), 6.04 (1H, s), 6.71—7.49 (4H, m).

4-(2-Bromoethoxy)benzaldehyde Ethylene Acetal (VIIe, *p*-Isomer, n=2, X=Br)——90% yield. bp 144—155 °C (0.4 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 3.10 (2H, t, J=6 Hz), 3.90—4.40 (6H, m), 5.72 (1H, s), 6.85, 7.39 (2H each, ABq, J=9 Hz).

4-(3-Chloropropoxy)benzaldehyde Ethylene Acetal (VIIf, *p*-Isomer, n=3, X=Cl)——85% yield. bp 151—156 °C (0.3 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 2.19 (2H, m), 3.69 (2H, t, J=6 Hz), 3.90—4.30 (7H, m), 5.72 (1H, s), 6.87, 7.38 (2H each, ABq, J=9 Hz).

2-(2-(4-Phenylpiperazino)ethoxy)benzaldehyde (IX_1)——Method C: A mixture of VIIa (18.3 g, 80 mmol), *N*-phenylpiperazine (13.6 g, 84 mmol), NaI (12.0 g, 80 mmol), and K_2CO_3 (12.2 g, 88 mmol) in DMF (200 ml) was stirred at 100 °C for 5 h under an argon atmosphere. The mixture was concentrated to one-third of the initial volume and poured into water. The liberated oil was extracted with AcOEt. The extracts were washed with water, dried, and evaporated *in vacuo*. The residue was dissolved in a mixture of tetrahydrofuran (THF) (50 ml) and 10% aq. HCl (30 ml). After being stirred for 1 h at room temperature, the mixture was made alkaline with 10% aq. NaOH, diluted with water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–benzene (1 : 1) and recrystallized from isopropyl ether to give 15.5 g of IX_1. IR ν_{max}^{Nujol} cm^{-1}: 1680. MS m/z: 310 (M^+). Compounds $IX_{2-5,7-14,16,17,19,21-25,28}$ were prepared in a similar manner and their physical properties are listed in Table II.

1-(2-Chloroethyl)-4-(3-fluorophenyl)piperazine (VIIIa, $n=2$, $R^1=$3-F, X=Cl)——Thionyl chloride (12 g) was added dropwise to a stirred solution of 4-(3-fluorophenyl)-1-piperazinoethanol[9] (5.90 g, 26.3 mmol) in $CHCl_3$ (120 ml). The mixture was refluxed for 1 h and evaporated to dryness. The residue was recrystallized from EtOH–Et_2O to give 6.70 g (91.2%) of the dihydrochloride of VIIIa as needles, mp 215—217 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 2000—2550, 1610, 1510. MS *m/z*: 242 (M^+), 206, 150. ^{1}H-NMR (D_2O) δ: 3.40—4.15 (12H, m), 6.75—7.55 (4H, m).

1-(3-Chlorophenyl)-4-(3-chloropropyl)piperazine (VIIIb, $n=3$, $R^1=$3-Cl, X=Cl)——A mixture of 4-(3-chlorophenyl)-1-piperazinopropanol[9] (2.0 g, 7.86 mmol) and triphenylphosphine (2.06 g, 7.86 mmol) in CCl_4 (20 ml) was refluxed overnight. After cooling, the precipitate was filtered off, and the filtrate was concentrated. The residue was chromatographed on silica and eluted with AcOEt–benzene (1 : 4) to give 1.50 g (70%) of VIIIb as an oil. MS *m/z*: 274, 272 (M^+), 211, 209. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.2 (2H, m), 2.4—2.9 (6H, m), 3.1—3.4 (4H, m), 3.60 (2H, t, $J=$ 6 Hz), 6.65—7.50 (4H, m). The following compounds were prepared in a similar manner.

1-(3-Chloropropyl)-4-(3-fluorophenyl)piperazine (VIIIc, $n=3$, $R^1=$3-F, X=Cl)——72% yield. Oil. ^{1}H-NMR ($CDCl_3$) δ: 1.84—2.15 (2H, m), 2.25—2.75 (6H, m), 3.04—3.45 (4H, m), 3.58 (2H, t, $J=6$ Hz), 6.30—7.45 (4H, m).

1-(3-Chloropropyl)-4-(3-methylphenyl)piperazine (VIIId, $n=3$, $R^1=$3-CH_3, X=Cl)——79% yield. Oil. ^{1}H-NMR ($CDCl_3$) δ: 1.90—2.29 (2H, m), 2.30 (3H, s), 2.35—2.80 (6H, m), 3.10—3.40 (4H, m), 3.61 (2H, t, $J=6$ Hz), 6.60—7.60 (4H, m).

1-(3-Chloropropyl)-4-phenylpiperazine (VIIIe, $n=3$, $R^1=$H, X=Cl)——74% yield. Oil. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.2 (2H, m), 2.3—2.8 (6H, m), 3.1—3.4 (4H, m), 3.61 (2H, t, $J=6$ Hz), 6.5—7.5 (5H, m).

2-(3-(4-(3-Chlorophenyl)piperazino)propoxy)benzaldehyde (IX_{15})——Method D: A mixture of salicylaldehyde (0.67 g, 5.5 mmol), VIIIb (1.50 g, 5.5 mmol), and K_2CO_3 (0.76 g) in DMF (10 ml) was stirred at 60 °C overnight. The mixture was diluted with water and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–benzene (1 : 4) and recrystallized from Et_2O–hexane to give 1.20 g of IX_{15}. IR ν_{max}^{Nujol} cm^{-1}: 1680. Compounds $IX_{6,18,20,26,27}$ were prepared in a similar manner and their physical properties are listed in Table II.

2-(2-(3-(4-(3-Chlorophenyl)piperazino)propoxy)phenyl)-*N*-methylthiazolidine-3-thiocarboxamide (II_{24})——Method E: A mixture of IV[1] (*o*-isomer, Y=S, 0.51 g, 2.2 mmol), VIIIb (0.60 g, 2.2 mmol), K_2CO_3 (0.28 g, 2 mmol), and NaI (0.29 g, 2 mmol) in DMF (10 ml) was stirred at 80 °C overnight. The mixture was concentrated, diluted with water, and extracted with AcOEt. The extracts were washed successively with 10% aq. NaOH and water, and evaporated. The residue was purified by silica gel chromatography with AcOEt–benzene (2 : 3) and recrystallized from AcOEt–hexane to give 0.51 g of II_{24} as needles. IR ν_{max}^{Nujol} cm^{-1}: 3400. MS *m/z*: 490 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.75—2.4 (2H, m), 2.5—2.81 (6H, m), 2.9—3.4 (9H, m), 4.0—4.7 (4H, m), 5.4 (1H, br), 6.31 (1H, s), 6.6—7.5 (8H, m).

***N*-Methyl-2-(2-(2-(4-(2-methylphenyl)piperazino)ethoxy)phenyl)thiazolidine-3-carboxamide (II_{20})**——Method F: A mixture of Vb (2.10 g, 7 mmol), 1-(2-methylphenyl)piperazine (1.23 g, 7 mmol), K_2CO_3 (0.97 g, 7 mmol), and NaI (1.05 g, 7 mmol) in DMF (20 ml) was heated at 80 °C for 20 h. After removal of the solvent, the residue was diluted with water and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–MeOH (40 : 1) and recrystallized from $CHCl_3$–Et_2O to give 2.26 g of II_{20}. IR ν_{max}^{Nujol} cm^{-1}: 3280, 1630. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s), 2.50—3.30 (15H, m), 3.50—4.80 (5H, m), 6.22 (1H, s), 6.80—7.50 (8H, m). MS *m/z*: 440 (M^+). Compounds $II_{15-17,19,21,22,29,36-39,42,43,45}$ were prepared in a similar manner and their properties are listed in Table III.

***N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-thiocarboxamide (II_1)**——Method G: This compound was prepared according to the reported procedure[1] from IX_1, cysteamine, and methyl isothiocyanate. IR ν_{max}^{Nujol} cm^{-1}: 3375. MS *m/z*: 442 (M^+), 369. ^{1}H-NMR ($CDCl_3$) δ: 2.69—3.33 (12H, m), 3.01 (3H, d, $J=4.5$ Hz), 4.24 (2H, t, $J=5$ Hz), 4.24—4.83 (2H, m), 5.56 (1H, br), 6.37 (1H, s), 6.70—7.42 (9H, m). Compounds $II_{2-14,18,23,25-28,30-35,40,41,44,50,51,55,58-61}$ were prepared in a similar manner and their properties are listed in Tables III and IV.

2-(2-(2-(4-Phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide (II_{46})——Method H: A solution of acetic acid (2.46 g, 41 mmol) in EtOH (5 ml) was added dropwise to a suspension of X (*o*-isomer, $n=2$, $R^1=$H; 3.03 g, 8.2 mmol, prepared from the corresponding benzaldehyde and cysteamine) and sodium cyanate (1.07 g, 16.4 mmol) in EtOH (40 ml). After being stirred for 2 h at room temperature, the mixture was made alkaline with 10% aq. K_2CO_3, concentrated, and extracted with AcOEt. The extracts were washed with water, dried over Na_2SO_4, and evaporated. The residue was recrystallized from AcOEt to give 2.22 g of II_{46}. IR ν_{max}^{Nujol} cm^{-1}: 1640. ^{1}H-NMR ($CDCl_3$) δ: 2.66—3.30 (12H, m), 3.57—4.43 (4H, m), 4.81 (2H, br s), 6.25 (1H, s), 6.83—7.38 (9H, m). MS *m/z*: 412 (M^+). Compound II_{49} was prepared in a similar manner and its properties are listed in Table IV.

***N,N*-Dimethyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide (II_{48})**——Method I: A mixture of X (*o*-isomer, $n=2$, $R^1=$H; 2.00 g, 5.4 mmol), dimethylcarbamoyl chloride (0.87 g, 8.1 mmol), and K_2CO_3 (1.19 g, 8.6 mmol) in DMF (20 ml) was heated at 50 °C for 5 h. The mixture was poured into water and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–benzene (5 : 1) and recrystallized from isopropyl ether to give 1.39 g of II_{48}. IR ν_{max}^{Nujol} cm^{-1}: 1620. ^{1}H-NMR ($CDCl_3$) δ: 2.68—3.74 (12H, m), 2.81 (6H, s), 3.94—4.58 (4H, m), 6.24 (1H, s), 6.69—7.32 (9H, m). Compound II_{54} was prepared in a similar manner and its properties are listed in Table IV.

***N,N*-Dimethyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-thiocarboxamide (II_{56})**——Method J: A solution of X (*o*-isomer, $n=2$, $R^1=H$; 1.60 g, 4.3 mmol) in THF (30 ml) was added to a stirred solution of thiophosgene (1.00 g, 8.6 mmol) in THF (20 ml) under ice-cooling over a period of 3 h, and then dimethylamine (1.56 g, 34.6 mmol) in toluene (15 ml) was added to the mixture. After being stirred for 30 min at room temperature, the mixture was poured into water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was chromatographed on silica gel and eluted with AcOEt–benzene (1 : 1) to give 0.90 g of II_{56} as an oil. IR ν_{max}^{film} cm^{-1}: 1600, 1500. ^{1}H-NMR ($CDCl_3$) δ: 2.70—3.65 (12H, m), 3.13 (6H, s), 3.75—3.89 (1H, m), 4.21 (2H, t, $J=6$ Hz), 4.45—4.90 (1H, m), 6.66—7.34 (10H, m). MS *m/z*: 456 (M^+). Compound II_{63} was prepared in a similar manner and its properties are listed in Table IV.

***N*-Acetyl-2-(2-(2-(4-(3-fluorophenyl)piperazino)ethoxy)phenyl)-*N*-methylthiazolidine-3-thiocarboxamide (II_{62})**——Method K: A solution of II_6 (1.90 g, 4.1 mmol) in DMF (10 ml) was added to a suspension of NaH (60% oil dispersion, 0.18 g, 4.5 mmol) in DMF (5 ml) under ice-cooling, and the mixture was stirred for 20 min under an argon atmosphere. A solution of acetyl chloride (0.36 g, 4.5 mmol) in Et_2O (10 ml) was added to the mixture, and the whole was stirred at room temperature overnight. After dilution with water, the mixture was extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was chromatographed on silica gel and eluted with AcOEt–hexane (1 : 1) to afford 1.60 g of II_{62} as an oil. IR ν_{max}^{film} cm^{-1}: 1670. ^{1}H-NMR ($CDCl_3$) δ: 2.13 (3H, s), 2.30—3.50 (15H, m), 4.00—4.80 (4H, m), 6.10—7.50 (9H, m). MS *m/z*: 502 (M^+). Compound II_{53} was prepared in a similar manner and its properties are listed in Table IV.

2-(2-(2-(4-(3-Fluorophenyl)piperazino)ethoxy)thiazolizine-3-thiocarboxamide (II_{57})——Method L: This compound was prepared according to the reported procedure[1] from II_{61} and 10% aq. NaOH. IR ν_{max}^{Nujol} cm^{-1}: 3430, 3310, 3180. ^{1}H-NMR ($CDCl_3$) δ: 2.60—3.40 (12H, m), 4.10—4.80 (4H, m), 5.76 (2H, brs), 6.35 (1H, s), 6.30—7.50 (8H, m).

***N*-Acetyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide (II_{47})**——Method M: A mixture of II_{46} (1.49 g, 3.5 mmol), triethylamine (0.55 g, 5.4 mmol), and acetyl chloride (0.42 g, 5.4 mmol) in benzene (60 ml) was heated at 80 °C for 1.5 h. An additional amount of triethylamine (0.55 g, 5.4 mmol) and acetyl chloride (0.42 g, 5.4 mmol) was added to the mixture, and the whole was heated at 80 °C for 1 h. The mixture was concentrated, diluted with water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–benzene (1 : 1) and recrystallized from $CHCl_3$–Et_2O to give 1.25 g (70%) of the diacetate of II_{46}[4] as crystals, mp 120—123.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1730, 1700, 1680. MS *m/z*: 496 (M^+), 368, 189, 175, 132. ^{1}H-NMR ($CDCl_3$) δ: 2.35 (6H, m), 2.67—2.32 (12H, m), 3.98—4.31 (4H, m), 6.85—7.47 (10H, m). The diacetate (1.19 g, 2.4 mmol) was hydrolyzed by treatment with a mixture of NaOH (0.29 g, 7.2 mmol), H_2O (2.7 ml), EtOH (20 ml), and THF (20 ml) under ice-cooling for 1.5 h. The mixture was concentrated, diluted with water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was purified by silica gel chromatography with AcOEt–$CHCl_3$ (5 : 1) and recrystallized from AcOEt–hexane to give 0.72 g (67%) of II_{47} as colorless prisms. IR ν_{max}^{Nujol} cm^{-1}: 3260, 1690, 1680, 1660, 1600. MS *m/z*: (M^+ was not observed) 369, 309, 237, 186, 175, 132. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (3H, s), 2.67—3.38 (12H, m), 3.72—4.49 (4H, m), 6.44 (1H, s), 6.88—7.44 (9H, m), 7.94 (1H, s). Compounds II_{52} was prepared in a similar manner from II_{49} and its properties are listed in Table IV.

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Synthesis of 2-Phenylthiazolidine Derivatives as Cardiotonic Agents. III.[1] Optically Active Isomers of *N*-Methyl-2-(2-(2-(4-phenylpiperazino)-ethoxy)phenyl)thiazolidine-3-carboxamides

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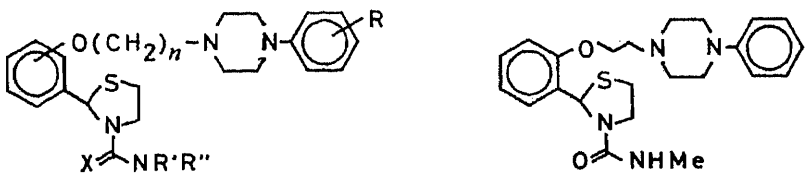
Optically active isomers of *N*-methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamides ((+)-**2** and (−)-**2**) have been synthesized and tested for positive inotropic activity. The racemic thiocarboxamide ((±)-**3**) was resolved into the enantiomers ((+)-**3** and (−)-**3**) *via* the L- and D-*N*-(2-naphthylsulfonyl)prolyl derivatives ((+)-**4** and (−)-**4**). Conversion of the thiocarboxamides ((+)-**3** and (−)-**3**) to the carboxamides ((+)-**2** and (−)-**2**) was smoothly effected by treatment with the glycidic ester (**7**). The absolute stereochemistry of (−)-**3** was determined to be 2*S* by X-ray crystallographic analysis. Hence, the absolute configuration of the carboxamide ((−)-**2**) is 2*S*. On i.v. administration to anesthetized dogs, the enantiomers of **2** showed only a threefold difference in positive inotropic activity, with the *levo* isomer ((−)-**2**) being the more active. In the isolated cat heart muscle, the enantiomers were nearly equipotent to each other. Thus, no significant difference between the positive inotropic activities of the optical isomers of **2** was observed.

Keywords——*N*-methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide; urea; optical resolution; 2-phenylthiazolidine; *N*-(2-naphthylsulfonyl)prolyl chloride; absolute stereochemistry; absolute configuration; X-ray crystallographic analysis; positive inotropic activity; cardiotonic agent

The preceding paper[1] of this series disclosed the synthesis and cardiotonic activity of novel 2-phenylthiazolidine derivatives represented by general formula (**1**). Among the large number of derivatives synthesized, *N*-methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)-phenyl)thiazolidine-3-carboxamide (**2**) was found to produce a marked and sustained positive inotropic action without producing significant alteration in heart rate or blood pressure in anesthetized dogs. In view of the potential usefulness of **2** as a new cardiotonic agent, the effect of its optical resolution on the activity was examined. We describe here the synthesis of optically active isomers of **2**, determination of their absolute stereochemistry by X-ray crystallographic analysis, and their cardiotonic activity.



1 : X=O, S 2

Chart 1

Chemistry

Several attempts to resolve the racemate ((±)-**2**) with optically active acids were without success. Efforts were then turned to the resolution of the corresponding thiocarboxamide ((±)-**3**)[1] *via* the optically active *N*-acyl derivative.[2] The racemate ((±)-**3**) was allowed to react with *N*-(2-naphthylsulfonyl)-L-prolyl chloride[3] (L-NSPCl) in the presence of NaH in dimethylformamide (DMF), giving a diastereoisomeric mixture of the *N*-acylthiocarboxamides ((+)-**4** and **5** (2*S*)) together with the aldehyde (**6**).[1] Chromatography of this mixture on silica gel gave (+)-**4**, mp 191—191.5 °C, in 24.1% yield.

The diastereoisomer (**5** (2*S*)) could not be isolated in a pure state, since attempts to separate it from the aldehyde (**6**) were unsuccessful. Alkaline hydrolysis of (+)-**4** at room temperature gave 93.3% yield of the thiocarboxamide ((+)-**3**), mp 144—144.5 °C, $[\alpha]_D^{20}$ +145.7°. Similar treatment of the crude **5** (2*S*) gave (−)-**3** in only low yield (4.1% from (±)-**3**). To obtain the *levo* isomer ((−)-**3**) in better yield, we carried out acylation of (±)-**3**

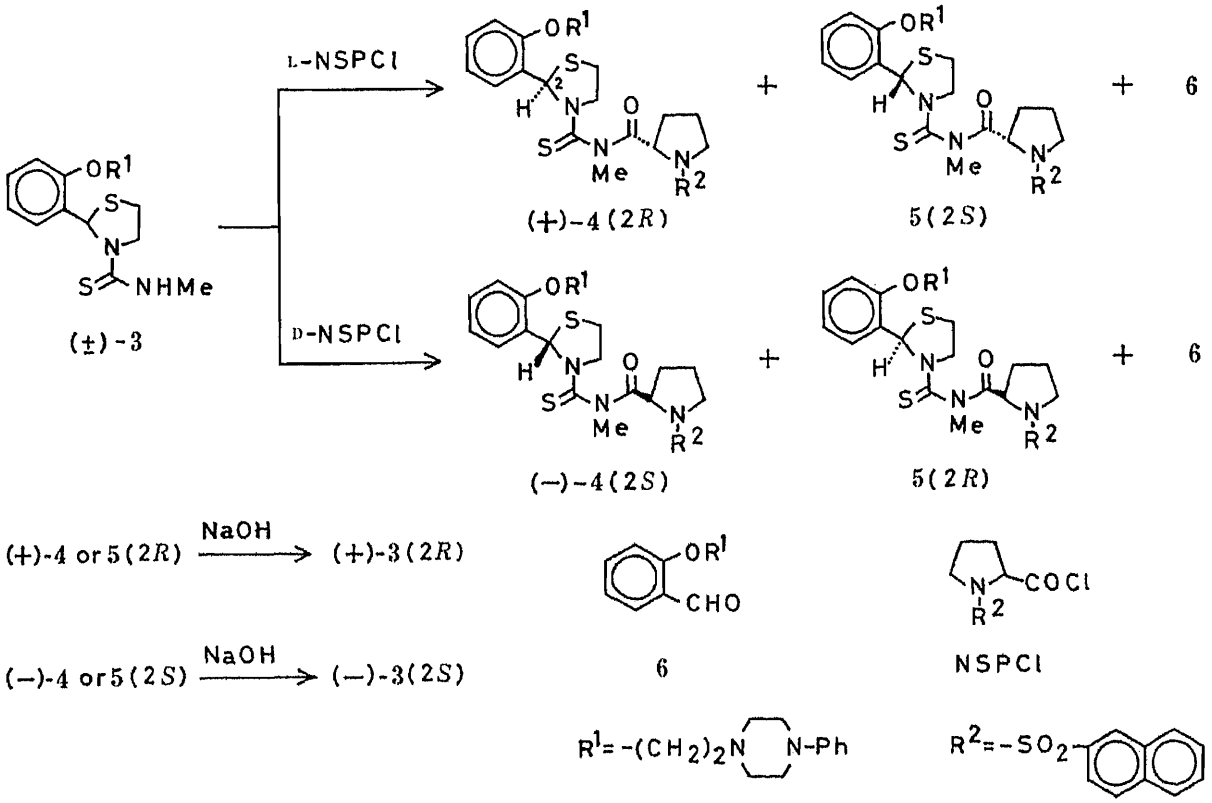


Chart 2

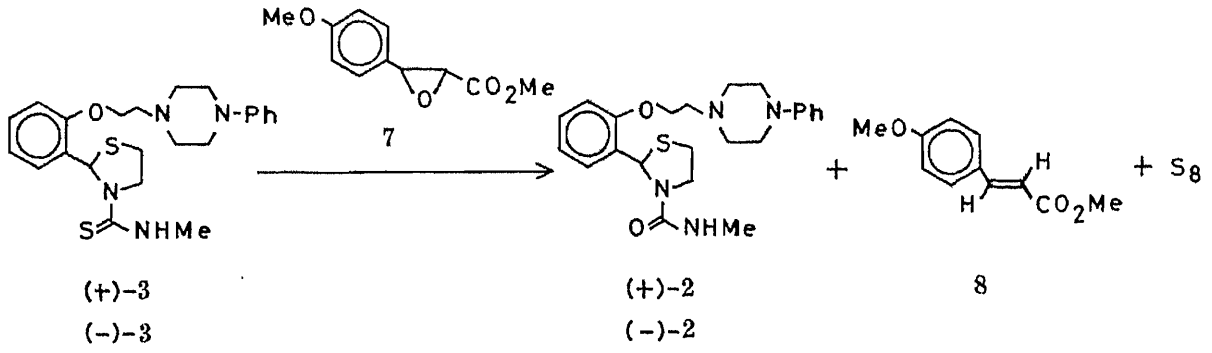


Chart 3

with *N*-(2-naphthylsulfonyl)-D-prolyl chloride (D-NSPCl), giving (−)-**4**, mp 191—192 °C, as a major product (24.6%). Alkaline hydrolysis of (−)-**4** gave (−)-**3**, mp 144—145.5 °C, $[\alpha]_D^{20}$ −145.3°, in 94.2% yield (Chart 2).

Conversion of the thiocarboxamides ((+)-**3** and (−)-**3**) to the corresponding carboxamides ((+)-**2** and (−)-**2**) was smoothly effected by treatment with methyl *trans*-3-(4-methoxyphenyl)glycidate (**7**).[4] Reaction of thioureas with oxiranes has been reported to give episulfides or olefins together with ureas *via* isothiuronium salts.[5] When heated with the glycidate (**7**) in EtOH, (+)-**3** readily gave the carboxamide ((+)-**2**), mp 151—152 °C, $[\alpha]_D^{20}$ +152.38°, in 75.7% yield after simple chromatographic separation from the methyl cinnamate (**8**) and elemental sulfur. Similarly, (−)-**2**, mp 151.5—152.5 °C, $[\alpha]_D^{20}$ −154.1°, was obtained from (−)-**3** in 76.7% yield. Various methods for oxidative conversion of thioureas

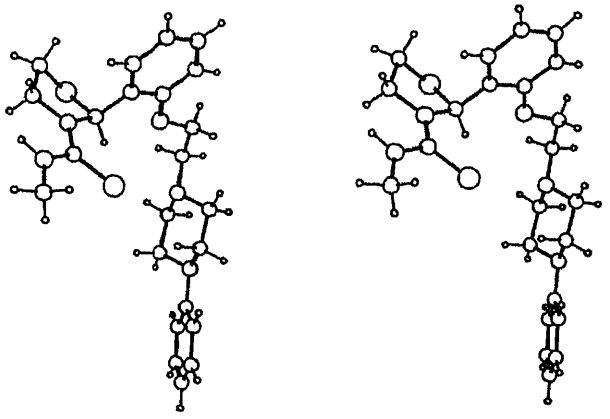
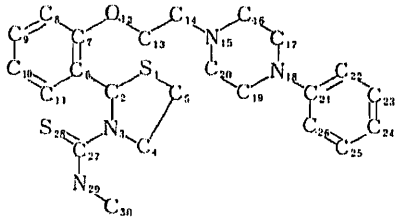


Fig. 1. Stereoscopic Drawing of (−)-3

TABLE 1. Fractional Coordinates and Isotropic Temperature Factors (Å²) with e.s.d.'s in Parentheses



Atom	*x*	*y*	*z*	B_{eq}	Atom	*x*	*y*	*z*	B_{eq}
S(1)	0.68920 (14)	0.54995 (6)	1.07069 (18)	5.61 (4)	C(16)	0.9153 (6)	0.8073 (3)	0.8987 (7)	6.6 (2)
C(2)	0.8504 (5)	0.5744 (2)	1.0420 (6)	4.6 (1)	C(17)	0.9813 (5)	0.8037 (3)	0.7506 (7)	7.1 (2)
N(3)	0.9129 (4)	0.5251 (1)	0.9825 (5)	4.7 (1)	N(18)	0.8987 (4)	0.8292 (2)	0.6400 (5)	5.3 (1)
C(4)	0.8519 (6)	0.4707 (2)	1.0125 (6)	5.4 (2)	C(19)	0.7811 (5)	0.8007 (3)	0.6342 (7)	6.3 (2)
C(5)	0.7465 (6)	0.4815 (2)	1.1191 (7)	5.6 (2)	C(20)	0.7192 (5)	0.8008 (2)	0.7854 (8)	6.2 (2)
C(6)	0.9035 (5)	0.5953 (2)	1.1852 (5)	4.4 (1)	C(21)	0.9526 (5)	0.8386 (2)	0.5009 (6)	4.9 (1)
C(7)	0.8789 (5)	0.6510 (2)	1.2287 (6)	4.6 (1)	C(22)	1.0769 (5)	0.8250 (2)	0.4679 (7)	5.3 (2)
C(8)	0.9195 (5)	0.6699 (2)	1.3661 (6)	5.3 (2)	C(23)	1.1270 (6)	0.8382 (2)	0.3328 (7)	6.2 (2)
C(9)	0.9845 (6)	0.6344 (3)	1.4576 (6)	6.1 (2)	C(24)	1.0556 (7)	0.8635 (2)	0.2255 (7)	6.8 (2)
C(10)	1.0116 (5)	0.5803 (2)	1.4156 (7)	5.9 (2)	C(25)	0.9346 (7)	0.8783 (2)	0.2573 (7)	6.8 (2)
C(11)	0.9718 (5)	0.5618 (2)	1.2792 (6)	5.3 (2)	C(26)	0.8827 (6)	0.8660 (2)	0.3920 (7)	6.1 (2)
O(12)	0.8137 (3)	0.6829 (1)	1.1311 (4)	5.3 (1)	C(27)	1.0149 (6)	0.5304 (2)	0.8965 (6)	5.3 (2)
C(13)	0.8062 (6)	0.7422 (2)	1.1591 (7)	5.5 (2)	S(28)	1.07698 (18)	0.59330 (6)	0.85690 (21)	7.10 (5)
C(14)	0.7380 (5)	0.7704 (2)	1.0367 (7)	5.6 (2)	N(29)	1.0629 (5)	0.4822 (2)	0.8430 (6)	6.3 (2)
N(15)	0.8021 (4)	0.7740 (2)	0.8971 (6)	5.6 (1)	C(30)	1.1740 (8)	0.4785 (3)	0.7530 (9)	8.8 (3)

$B_{eq} = 4/3 \sum_i \sum_j \beta_{ij} \boldsymbol{a}_i \cdot \boldsymbol{a}_j$.

TABLE II. Bond Angles (°) with e.s.d's in Parentheses

Bond		Bond		Bond	
C(2)–S(1)–C(5)	89.9 (2)	C(7)–C(8)–C(9)	120.2 (5)	N(18)–C(19)–C(20)	110.4 (5)
S(1)–C(2)–N(3)	103.4 (3)	C(8)–C(9)–C(10)	120.8 (5)	N(15)–C(20)–C(19)	110.8 (5)
S(1)–C(2)–C(6)	109.6 (4)	C(9)–C(10)–C(11)	118.8 (5)	N(18)–C(21)–C(22)	123.0 (5)
N(3)–C(2)–C(6)	114.5 (4)	C(6)–C(11)–C(10)	122.5 (5)	N(18)–C(21)–C(26)	119.3 (5)
C(2)–N(3)–C(4)	115.6 (4)	C(7)–O(12)–C(13)	117.4 (4)	C(22)–C(21)–C(26)	117.5 (5)
C(2)–N(3)–C(27)	120.9 (4)	O(12)–C(13)–C(14)	109.6 (4)	C(21)–C(22)–C(23)	120.6 (5)
C(4)–N(3)–C(27)	123.3 (4)	C(13)–C(14)–N(15)	116.7 (5)	C(22)–C(23)–C(24)	121.0 (6)
N(3)–C(4)–C(5)	107.5 (4)	C(14)–N(15)–C(16)	114.9 (5)	C(23)–C(24)–C(25)	119.1 (6)
S(1)–C(5)–C(4)	104.7 (4)	C(14)–N(15)–C(20)	109.9 (4)	C(24)–C(25)–C(26)	121.0 (6)
C(2)–C(6)–C(7)	119.0 (4)	C(16)–N(15)–C(20)	105.9 (4)	C(21)–C(26)–C(25)	120.8 (6)
C(2)–C(6)–C(11)	123.2 (4)	N(15)–C(16)–C(17)	110.5 (5)	N(3)–C(27)–S(28)	122.0 (4)
C(7)–C(6)–C(11)	117.8 (5)	C(16)–C(17)–N(18)	107.7 (5)	N(3)–C(27)–N(29)	116.0 (4)
C(6)–C(7)–C(8)	119.8 (5)	C(17)–N(18)–C(19)	111.1 (5)	S(28)–C(27)–N(29)	121.9 (4)
C(6)–C(7)–O(12)	115.8 (4)	C(17)–N(18)–C(21)	115.7 (4)	C(27)–N(29)–C(30)	124.7 (5)
C(8)–C(7)–O(12)	124.4 (4)	C(19)–N(18)–C(21)	113.7 (5)		

TABLE III. Bond Distance (Å) with e.s.d.'s in Parentheses

Bond		Bond	
S(1)–C(2)	1.841 (6)	N(15)–C(16)	1.449 (7)
S(1)–C(5)	1.792 (5)	N(15)–C(20)	1.493 (8)
C(2)–N(3)	1.453 (6)	C(16)–C(17)	1.525 (9)
C(2)–C(6)	1.507 (7)	C(17)–N(18)	1.471 (8)
N(3)–C(4)	1.474 (6)	N(18)–C(19)	1.432 (7)
N(3)–C(27)	1.351 (7)	N(18)–C(21)	1.409 (7)
C(4)–C(5)	1.512 (9)	C(19)–C(20)	1.528 (9)
C(6)–C(7)	1.405 (6)	C(21)–C(22)	1.404 (8)
C(6)–C(11)	1.379 (7)	C(21)–C(26)	1.403 (8)
C(7)–C(8)	1.399 (8)	C(22)–C(23)	1.378 (9)
C(7)–O(12)	1.361 (6)	C(23)–C(24)	1.379 (9)
C(8)–C(9)	1.375 (8)	C(24)–C(25)	1.375 (10)
C(9)–C(10)	1.372 (8)	C(25)–C(26)	1.378 (9)
C(10)–C(11)	1.385 (8)	C(27)–S(28)	1.674 (5)
O(12)–C(13)	1.434 (6)	C(27)–N(29)	1.348 (7)
C(13)–C(14)	1.492 (8)	N(29)–C(30)	1.448 (10)
C(14)–N(15)	1.447 (8)		

TABLE IV. Determination of the Absolute Configuration

h	k	l	$\lvert F_o(hkl)\rvert / \lvert F_o(h\bar{k}\bar{l})\rvert$	$\lvert F_c(hkl)\rvert / \lvert F_c(h\bar{k}\bar{l})\rvert$
1	5	1	0.932	0.915
2	4	1	0.940	0.931
2	5	1	1.066	1.048
2	8	1	0.921	0.939
3	1	1	1.096	1.086
5	7	1	0.924	0.954
1	5	2	0.929	0.970
1	6	2	1.092	1.061
2	5	2	1.063	1.054
2	12	2	0.926	0.930
3	3	2	1.090	1.045
3	5	2	1.086	1.081
3	10	2	1.058	1.060
6	5	2	0.934	0.965
2	6	3	0.943	0.968
3	2	3	0.903	0.893
3	6	3	1.058	1.045

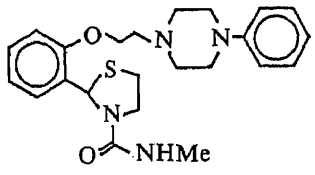
into ureas have been reported recently.[6] The above procedure, however, appears to be of practical use in view of the mild and neutral reaction conditions (Chart 3).

The absolute stereochemistry of the thiocarboxamide ((−)-**3**) was determined to be 2*S* by X-ray crystallographic analysis, and a stereoscopic drawing of the molecule is shown in Fig. 1. It follows from this fact that the absolute configurations of the carboxamides ((−)-**2** and (+)-**2**) are 2*S* and 2*R*, respectively.

Pharmacology

The optically active isomers of the carboxamide (**2**) were tested for positive inotropic activity by measuring the increase in the maximum derivative of left ventricular pressure ($LVdP/dt_{max}$) after i.v. administration to anesthetized dogs by the method reported previously.[7] The results are summarized in Table V together with comparative data for the

TABLE V. *N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide ((+)-**2**, (−)-**2**, and (±)-**2**)



Compd.	Myocardial contractility Anesthetized dog[a]		
	Dose (mg/kg) i.v.	LV$dP/dt_{max}$ (Δ %)	Duration (min)
(+)-**2** (2*R*) Oxalate	0.01	20	30
(−)-**2** (2*S*) Oxalate	0.003	36	40
(±)-**2** Oxalate	0.003	30	30

a) For methodology, see reference 7.

racemate (**2**).[1)]

The positive inotropic activity of **2** was not significantly changed by optical resolution. Its enantiomers showed only an approximately threefold difference in activity, with the *levo* isomer ((−)-**2**) being the more active. The *levo* isomer ((−)-**2**) was nearly equipotent to the racemate. In the isolated cat heart muscle,[8)] the optical isomers of **2** produced a dose-dependent increase in contractile force from 4×10^{-7} M. The enantiomers, however, were nearly equipotent to each other in this test also. The rather uniform activity of the enantiomers of **2** appears to suggest that the positive inotropic activity of **2** is largely conferred by the phenylpiperazinoethoxy moiety[1)] and that the change in the chirality at C_2 of the thiazolidine ring, which is remote from this moiety, does not significantly alter the activity.

Further studies on 2-phenylthiazolidine derivatives as new cardiotonic agents are being continued.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded in Nujol mulls on a Hitachi IR-215 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken in $CDCl_3$ at 60 MHz on a JEOL PMX-60 spectrometer with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument. Optical rotations were determined for solutions in $CHCl_3$ or MeOH on a Union PM-201 automatic digital polarimeter.

Reaction of (±)-3 with L-NSPCl——The racemate ((±)-**3**, 5.33 g, 0.012 mol) was added to a stirred suspension of NaH (0.64 g, 50% oil dispersion, 0.013 mol) in DMF (55 ml) under ice-cooling, and the mixture was stirred for 0.5 h. A solution of L-NSPCl[3)] (4 g, 0.0124 mol) in DMF (40 ml) was added dropwise to the mixture under ice-cooling, and the whole was stirred at room temperature for 2 h. The mixture was diluted with ice-water and extracted with AcOEt. The AcOEt extracts were washed with sat. $NaHCO_3$ and water, dried, and evaporated to give an oil. The oil was chromatographed over SiO_2 and eluted with benzene–AcOEt (3 : 2). (*R*)-*N*-Methyl-*N*-(*N*-(2-naphthylsulfonyl)-L-propyl)-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-thiocarboxamide ((+)-**4**, 0.94 g, mp 191—191.5 °C from AcOEt–hexane) was obtained as needles from the first eluate. $[\alpha]_D^{20}$ +26.4° (c = 0.417, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 1690, 1600, 1490, 1345, 1150, 760. MS *m/z*: 729 (M^+), 597, 538, 469, 412, 368, 326, 292, 260 (base peak), 212, 191, 189, 175, 173, 160, 132, 128, 127. NMR ($CDCl_3$) δ: 1.5—2.4 (4H, m), 2.4—3.8 (17H, m), 4.0—4.2 (4H, m), 4.9 (1H, br), 6.7—7.4 (10H, m), 7.5—8.6 (6H, m), 8.44—8.55 (1H, m). *Anal.* Calcd for $C_{38}H_{43}N_5O_4S_3$: C, 62.53; H, 5.94; N, 9.59; S, 13.18. Found: C, 62.64; H, 5.99; N, 9.48; S, 13.02.

The following eluates containing (±)-**3**, (+)-**4**, **5** (2*S*), and **6** were evaporated and chromatographed over SiO_2 (benzene–AcOEt = 3 : 2) again. The first eluate gave an additional amount of (+)-**4** (0.12 g, total yield 24.1%, mp 190.5—191.5 °C) after recrystallization from AcOEt–hexane. From the second eluate, a mixture (0.2 g) of (+)-**4** and **5** (2*S*) was obtained. The third eluate gave a mixture (0.88 g) of **5** (2*S*) and the aldehyde (**6**) (see below). The starting material ((±)-**3**, 2.0 g (37.5%), mp 126—128 °C) was recovered from the fourth eluate after recrystallization from AcOEt–hexane.

D-NSPCl——This compound was obtained from D-proline in two steps (68%) by the same procedure as described for the L-isomer.[3] Recrystallization from a mixture of benzene–hexane gave needles, mp 102—104 °C. $[\alpha]_D^{20}$ +81.0° (c = 1, $CHCl_3$).

Reaction of (±)-3 with D-NSPCl——A mixture of (±)-**3** (5.33 g, 0.012 mol), D-NSPCl (4 g, 0.0124 mol), and NaH (0.64 g, 50% oil dispersion, 0.013 mol) was allowed to react and worked up in the same manner as described above to give 1.08 g (24.6%) of (−)-**4**, mp 191—192 °C (from AcOEt–hexane). $[\alpha]_D^{20}$ −26.5° (c = 0.2, $CHCl_3$). The spectral data of this compound were identical to those of (+)-**4**. A mixture (1.04 g) of the diastereoisomer (**5** (2*R*)) and the aldehyde (**6**) was also obtained from the following eluates.

(*R*)-*N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-thiocarboxamide ((+)-3)——a) Hydrolysis of (+)-**4**: A mixture of (+)-**4** (1.06 g, 0.00145 mol), NaOH (0.116 g, 0.0029 mol), H_2O (3 ml), MeOH (40 ml), and tetrahydrofuran (THF) (40 ml) was stirred at room temperature for 15.5 h and concentrated *in vacuo*. The residue was diluted with water and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were washed with H_2O, dried, and evaporated. The residue was purified by SiO_2 chromatography (benzene–AcOEt = 3 : 2) and recrystallized from AcOEt–hexane to give 0.6 g (93.3%) of (+)-**3**, mp 144—144.5 °C as prisms. $[\alpha]_D^{20}$ +145.7° (c = 0.365, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3420, 1600, 1525. MS *m/z*: 442 (M^+), 369, 309, 237, 186, 175, 132. NMR ($CDCl_3$) δ: 2.66—3.24 (12H, m), 3.02 (3H, d, J = 4.4 Hz), 4.19—4.70 (4H, m), 5.50 (1H, br), 6.33 (1H, s), 6.78—7.39 (9H, m). *Anal.* Calcd for $C_{23}H_{30}N_4OS_2$: C, 62.41; H, 6.83; N, 12.66; S, 14.49. Found: C, 62.39; H, 6.80; N, 12.56; S, 14.35. The fumarate was crystallized from Me_2CO–hexane and had mp 138—144 °C (dec.). $[\alpha]_D^{20}$ +65.0° (c = 0.150, MeOH). *Anal.* Calcd for $C_{23}H_{30}N_4OS_2 \cdot C_4H_4O_4 \cdot 0.5H_2O$: C, 57.12; H, 6.21; N, 9.87; S, 11.29. Found: C, 56.94; H, 6.12; N, 9.74; S, 11.52. From the aqueous layer, 0.4 g (90.2%) of *N*-(2-naphthylsulfonyl)-L-proline[3] (mp 133—135 °C, $[\alpha]_D^{20}$ −105.3°) was recovered.

b) Hydrolysis of Crude **5** (2*R*): A solution of the crude mixture (1.04 g) of **5** (2*R*) and **6** described above and NaOH (0.12 g) in H_2O (3 ml), THF (30 ml), and MeOH (30 ml) was stirred at room temperature for 45 h. The mixture was worked up as described above, and the crude product was purified by column chromatography (SiO_2, benzene–AcOEt = 3 : 2). The first eluate gave the aldehyde (**6**) (0.13 g, 3.5% from (±)-**3**) as an oil. The IR spectrum and MS of this compound were identical to those of an authentic sample.[1] The second eluate gave, after recrystallization from a mixture of AcOEt and hexane, 0.205 g (7.7% from (±)-**3**) of (+)-**3**, mp 144—145.5 °C. $[\alpha]_D^{20}$ +141.74° (c = 0.206, $CHCl_3$). Its spectral data were identical to those of (+)-**3** obtained from (+)-**4**.

(*S*)-*N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-thiocarboxamide ((−)-3)——a) Hydrolysis of (−)-**4**: A mixture of (−)-**4** (1.05 g, 0.00144 mol), NaOH (0.123 g, 0.00308 mol), H_2O (3 ml), MeOH (40 ml), and THF (40 ml) was hydrolyzed in the same manner as described for (+)-**3** to give 0.6 g (94.2%) of (−)-**3**, mp 144—145.5 °C (from AcOEt–hexane). $[\alpha]_D^{20}$ −145.37° (c = 0.216, $CHCl_3$). The spectral data were identical to those of (+)-**3**. *Anal.* Calcd for $C_{23}H_{30}N_4OS_2$: C, 62.41; H, 6.83; N, 12.66; S, 14.49. Found: C, 62.52; H, 6.93; N, 12.66; S, 14.52. The fumarate was crystallized from Me_2CO–hexane and had mp 138—144 °C (dec.). $[\alpha]_D^{20}$ −67.27° (c = 0.11, MeOH). *Anal.* Calcd for $C_{23}H_{30}N_4OS_2 \cdot C_4H_4O_4 \cdot 0.5H_2O$: C, 57.12; H, 6.21; N, 9.87; S, 11.29. Found: C, 56.91; H, 6.14; N, 9.70; S, 11.19.

b) Hydrolysis of Crude **5** (2*S*): The crude mixture (0.88 g) of **5** (2*S*) and **6** described above was hydrolyzed in a manner similar to that described above. Chromatographic separation and recrystallization from AcOEt–hexane gave 0.108 g (4.1% from (±)-**3**) of (−)-**3**, mp 142—144 °C. $[\alpha]_D^{20}$ −139.8° (c = 0.265, $CHCl_3$). The spectral data were identical to those of (−)-**3** obtained from (−)-**4**. The aldehyde (**6**, 0.16 g) was also isolated as an oil.

(*R*)-*N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide ((+)-2)——A solution of (+)-**3** (0.48 g, 0.00108 mol) and the glycidate (**7**, 0.339 g, 0.00162 mol) in EtOH (40 ml) was refluxed for 11 h. Additional **7** (0.339 g, 0.00162 mol) was added to the mixture, and the whole was refluxed for 3 h and then evaporated. The residue was chromatographed over SiO_2 and eluted with $CHCl_3$–EtOH (40 : 1). Elemental sulfur (23 mg (66%), mp 111—113 °C from ethanol) was obtained as needles from the first eluate. The second eluate gave, after recrystallization from a mixture of AcOEt and hexane, 0.11 g (50.5%) of **8**, mp 89—90 °C. IR ν_{max}^{Nujol} cm^{-1}: 1715, 1640. MS *m/z*: 192 (M^+). From the third eluate, 0.35 g (75.7%) of (+)-**2** was obtained as needles, mp 151—152 °C (from AcOEt–hexane). $[\alpha]_D^{20}$ +152.38° (c = 0.21, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3370, 1620, 1600, 1525, 760. NMR ($CDCl_3$) δ: 2.65—3.40 (15H, m), 3.65—4.60 (4H, m), 6.18 (1H, s), 6.70—7.40 (10H, m). MS *m/z*: 426 (M^+), 368. *Anal.* Calcd for $C_{23}H_{30}N_4O_2S$: C, 64.76; H, 7.09; N, 13.13; S, 7.52. Found: C, 64.60; H, 7.05; N, 13.20; S, 7.57. The oxalate was crystallized from Me_2CO and had mp 170—172 °C (dec.). $[\alpha]_D^{20}$ +88.63° (c = 0.102, MeOH). *Anal.* Calcd for $C_{23}H_{30}N_4O_2S \cdot C_2H_2O_4 \cdot 0.33H_2O$: C, 57.46; H, 6.30; N, 10.72; S, 6.13. Found: C, 57.36; H, 6.34; N, 10.53; S, 5.96.

(*S*)-*N*-Methyl-2-(2-(2-(4-phenylpiperazino)ethoxy)phenyl)thiazolidine-3-carboxamide ((−)-2)——Reaction of (−)-**3** (0.48 g, 0.00108 mol) with **7** (0.678 g, 0.00324 mol) in the same manner as described above gave 0.355 g (76.7%)

of (−)-**2**, mp 151.5—152.5 °C (from AcOEt–hexane). $[\alpha]_D^{20}$ −154.11° (c=0.23, $CHCl_3$). The spectral data were identical to those of (+)-**2**. The oxalate crystallized from Me_2CO had mp 170—172 °C (dec.). $[\alpha]_D^{20}$ −90.87° (c=0.130, MeOH). *Anal.* Calcd for $C_{23}H_{30}N_4O_2S \cdot C_2H_2O_4 \cdot 0.33H_2O$: C, 57.46; H, 6.30; N, 10.72; S, 6.13. Found: C, 57.64; H, 6.37; N, 10.54; S, 5.94.

Crystal Data for (−)-3——$C_{23}H_{30}N_4OS_2$, M_r=442.65, orthorhombic, $P2_12_12_1$, a=10.722 (1), b=23.749 (2), c=9.104 (1) Å, V=2318.1 (3) Å^3, D_c=1.268 g/cm^3, Z=4.

X-Ray Analysis——Colorless, plate-like crystals of (−)-**3** were obtained from ethanol solution by slow evaporation. Intensity data were measured on an automated diffractometer (Rigaku AFC-5) with graphite-monochromated CuK_α radiation. In total, 2272 reflections were measured, of which 1972 were judged significant ($|F_o| \geq 2.67\sigma(F_o)$). The structure was solved by the direct method using MULTAN 80[9] and refined by the block-diagonal least-squares with anisotropic temperature factors for all non-hydrogen atoms and with isotropic ones for all hydrogen atoms. The final R value was 0.058. The final atomic parameters, bond angles, and bond distances are listed in Tables I, II, and III, respectively.

Absolute Configuration——The absolute configuration was determined by the use of the anomalous dispersion term in the atomic scattering factor of the sulfur atom ($\Delta f'$=0.319, $\Delta f''$=0.557; International Tables for X-Ray Crystallography).[10] The observed and calculated intensity ratios for some Bijvoet pairs are listed in Table IV. The results in Table IV indicate that the absolute configuration of the molecule is 2*S*.

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Un Agent Trichomonacide: Le (Thénoyl-2')-amino-2 nitro-5 thiazole $C_8H_5O_3N_3S_2$. II. Structure Cristalline et Moleculaire d'une Nouvelle Variété Monoclinique Solvatée par le Diméthylformamide

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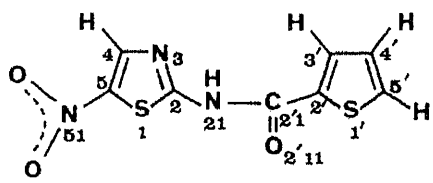
$C_8H_5O_3N_2S_2 \cdot C_3H_7NO$ crystallizes in dimethylformamide as space group $P2_1/n$ with lattice parameters $a=10.370(3)$, $b=8.180(8)$, $c=17.90(1)$ Å, $\beta=104.30(4)°$, $V=1471$ Å^3, $Z=4$, $D_m=1.56(2)$, $D_x=1.70$ Mg·m^{-3}. X-Ray data were collected on an ENRAF-NONIUS CAD-4 diffractometer using Mo$K\alpha$ radiation. The crystal structure was solved by direct methods and refined by full matrix least-squares analysis to a final R of 0.049 for 1783 independent reflections. The molecular geometry does not exhibit significant differences between the type I crystal previously described (without the dimethylformamide molecule as solvate) and the type II now studied. The crystal packing is reinforced by N–H···O hydrogen bonding of the amidic group and the DMF molecule. However, in the crystals of type II, the overlapping between adjacent molecules is not observed, contrary to the crystals of type I.

Keywords——crystal structure analysis; (2-thenoyl)-2-amino-5-nitrothiazole; dimethylformamide-solvate; trichomonacide

Dans un précédent mémoire,[1] nous avons décrit la structure cristalline et moléculaire de la variété monoclinique I du (thénoyl-2')-amino-2 nitro-5 thiazole (Tenonitrozole, Atrican*), agent trichomonacide doué de propriétés antimycotiques. Cette forme a été obtenue par recristallisation dans l'acétone.



Au cours des essais de cristallisation dans divers solvants, une nouvelle variété a pu être mise en évidence lors de l'utilisation du diméthylformamide (DMF) et de la méthyléthylcétone (MEC) dans un rapport volumique 1/2. Dans ce cas, les cristaux formés de couleur jaune paille, présentent une instabilité à l'air et leur étude nécessite l'emploi de tubes de Lindemann contenant le monocristal en présence de sa solution mère. D'autre part, les cristaux de cette

même variété ont été obtenus dans le DMF pur; ils ont été utilisés pour la détermination de la structure cristalline.

Détermination de la Structure Cristalline

Le cristal choisi mesure approximativement $350 \times 250 \times 200\,\mu m$. Les paramètres de la maille élémentaire

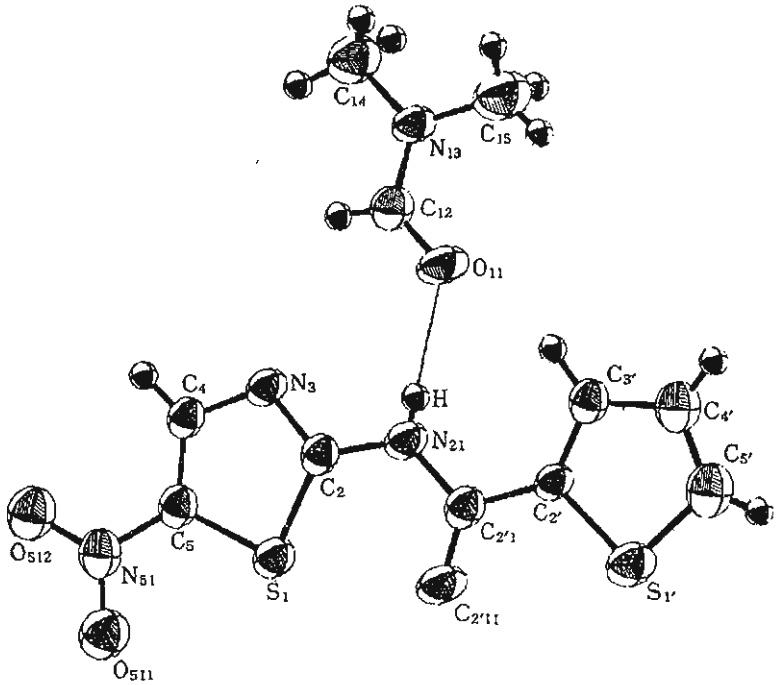


Fig. 1. Dessin de l'Unité Asymétrique

Les ellipsoïdes d'agitation thermique correspondent à 50% de probabilité.

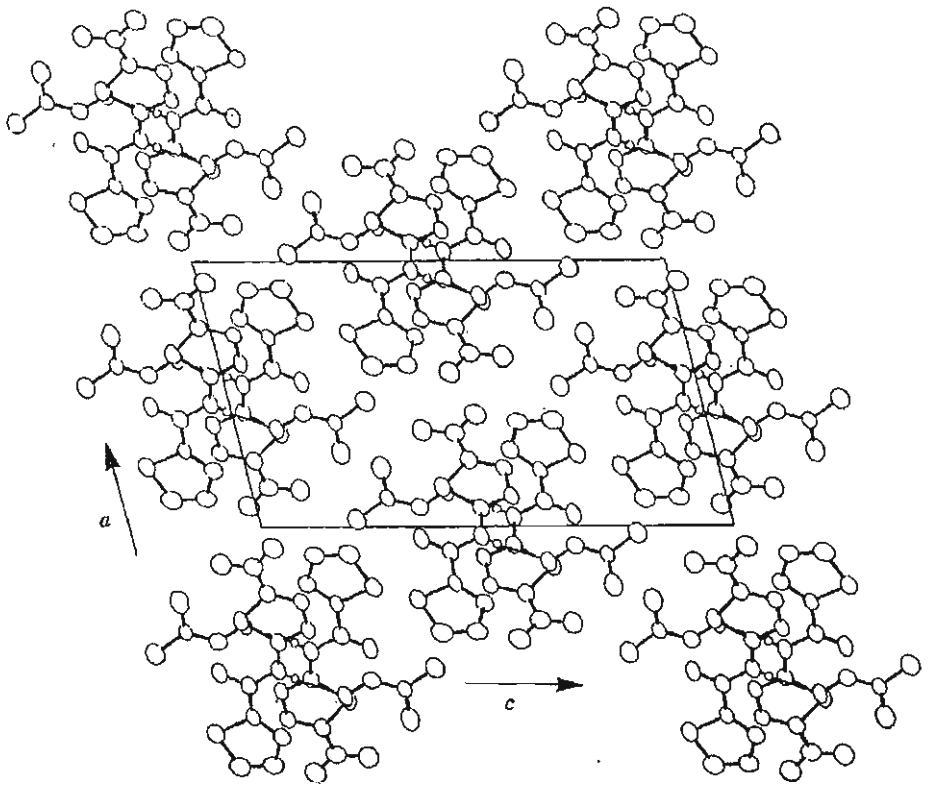


Fig. 2. Projection du Contenu de la Maille Parallèlement à b

déterminés à partir de 25 réflexions avec le rayonnement Mo$K\alpha$ ($15<\theta<16°$) ont pour valeur $a=10{,}370$ (3), $b=8{,}180$ (8), $c=17{,}90$ (1) Å, $\beta=104{,}30$ (4)°, $V=1471$ Å^3, $Z=4$, $D_m=1{,}56$ (2), $D_x=1{,}70$ Mg·m^{-3}, $\mu=0{,}331$ mm^{-1} (Mo$K\alpha$), $M_r=453{,}2$; $F=(000)=680$; groupe spatial $P2_1/n$.

Les intensités ont été mesurées sur un diffractomètre automatique CAD-4 ENRAF-NONIUS, avec un balayage θ–2θ, d'amplitude $s°=1{,}5+0{,}34\ \mathrm{tg}\,\theta$ ($1<\theta<23°$). Les réflexions 4 $\bar{5}$ $\bar{6}$ et $\bar{1}$ 0 13 ont été choisies pour contrôler les intensités. 1783 réflexions indépendantes ont été mesurées, 1255 réflexions d'entre elles répondant au critère $I>3\sigma(I)$ ont été retenues pour l'affinement. Aucune correction d'absorption n'a été effectuée.

La structure a été résolue par les méthodes directes à l'aide de MULTAN 80.[2] L'affinement basé sur F a été effectué par la méthode des moindres carrés à l'aide de programmes à matrice complète,[3] les facteurs de diffusion corrigés des f' et f'' sont issus de International Tables of Crystallography[4]; au cours de l'affinement, il a été tenu compte des corrections réelles de diffusion anomale pour S, C, N et O. Les positions des atomes d'hydrogène ont été localisées avec la série de Fourier des différences F_o-F_c puis affinées. Le facteur d'agitation thermique de l'atome d'hydrogène a été laissé fixe et égal à 4,7 Å^2. Les valeurs obtenues à l'issue du dernier affinement sont $R=0{,}049$ et $R_w=0{,}049$. Le schéma de pondération correspond à $w=1/\sigma^2(F)$. La valeur maximale du rapport de la variation Δ (sur les paramètres variables) sur l'écart-type σ correspond à 0,53; la valeur maximale de la densité électronique dans la série de Fourier finale des ΔF vaut 0,15 eÅ^{-3}.

Les distances, les angles valentiels et les distances aux plans moyens ont été calculés à l'aide du programme ORFFE3.[5] Le dessin de l'unité asymétrique (Fig. 1) et la projection de la maille parallèlement à l'axe $\vec{b}$ (Fig. 2) ont été obtenus à l'aide du programme ORTEP.[6]

TABLEAU I. Coordonnées Atomiques Relatives avec Écarts-Type et Facteurs de Température Équivalents

Atomes	x	y	z	$B_{éq}$ (Å^2) ou B_{iso}
S(1)	0,8510 (2)	0,3683 (3)	0,5934 (1)	3,33 (6)
C(2)	0,9306 (7)	0,3057 (9)	0,5247 (4)	2,9 (2)
N(3)	0,8844 (6)	0,3649 (8)	0,4545 (3)	3,1 (2)
C(4)	0,7796 (8)	0,465 (1)	0,4538 (4)	3,0 (2)
C(5)	0,7486 (8)	0,483 (1)	0,5225 (4)	3,1 (2)
N(51)	0,6494 (6)	0,5850 (9)	0,5378 (4)	3,8 (2)
O(511)	0,5786 (6)	0,6652 (8)	0,4848 (3)	4,7 (2)
O(512)	0,6386 (6)	0,5901 (8)	0,6046 (3)	4,6 (2)
N(21)	1,0359 (7)	0,2012 (9)	0,5394 (3)	3,2 (2)
C(2'1)	1,0936 (7)	0,142 (1)	0,6118 (4)	3,3 (2)
O(2'11)	1,0451 (5)	0,1728 (7)	0.6663 (3)	4,0 (2)
S(1')	1,2725 (2)	−0,0587 (3)	0,7069 (1)	4,29 (7)
C(2')	1,2115 (7)	0,0380 (9)	0,6197 (4)	2,9 (2)
C(3')	1,2864 (8)	0,003 (1)	0,5684 (4)	3,2 (2)
C(4')	1,3921 (8)	−0,103 (1)	0,6002 (5)	4,1 (3)
C(5')	1,3965 (9)	−0,147 (1)	0,6739 (5)	4,6 (3)
O(11)	1,1329 (5)	0,1554 (7)	0,4121 (3)	4,1 (2)
C(12)	1,0734 (8)	0,198 (1)	0,3473 (5)	3,5 (3)
N(13)	1,1079 (6)	0,1553 (8)	0,2840 (3)	3,2 (2)
C(14)	1,027 (1)	0,197 (2)	0,2080 (5)	5,0 (3)
C(15)	1,221 (1)	0,049 (2)	0,2882 (6)	5,7 (4)
HC(4)	0,743 (7)	0,52 (1)	0,408 (4)	4,7
H(N21)	1,066 (9)	0,20 (1)	0,509 (5)	4,7
H(C3')	1,274 (7)	0,06 (1)	0,519 (4)	4,7
H(C4')	1,462 (8)	−0,13 (1)	0,578 (4)	4,7
H(C5')	1,446 (8)	−0,21 (1)	0,705 (5)	4,7
H(C12)	1,001 (8)	0,26 (1)	0,336 (5)	4,7
H(C141)	0,953 (8)	0,26 (1)	0,212 (5)	4,7
H(C142)	1,081 (8)	0,22 (1)	0,186 (5)	4,7
H(C143)	0,997 (8)	0,08 (1)	0,187 (4)	4,7
H(C151)	1,193 (9)	−0,06 (1)	0,276 (5)	4,7
H(C152)	1,268 (8)	0,10 (1)	0,256 (5)	4,7
H(C153)	1,258 (8)	0,01 (1)	0,330 (5)	4,7

Resultats et Discussions

Les coordonnées des atomes et leurs facteurs d'agitation thermique isotrope équivalents sont rassemblés dans le Tableau I. Les distances et angles de liaisons sont indiqués sur le Tableau II qui précise la numérotation adoptée pour désigner les atomes situés dans la même unité asymétrique. L'analyse élémentaire montre l'existence d'une molécule de solvate pour une molécule citée en titre. L'analyse thermogravimétrique indique une perte en poids d'environ 20% entre 67 et 125 °C. Cette valeur est en bon accord avec une perte théorique de 23,25% correspondant à l'élimination d'une molécule de DMF en tant que solvate. Les spectres infrarouge (IR) d'un échantillon chauffé jusqu'à 133 °C montrent également des modifications qui peuvent être attribuées à l'élimination d'une molécule de DMF. Par ailleurs, on peut souligner que la comparaison des spectres IR du (thénoyl-2')-amino-2 nitro-5 thiazole, du DMF et du dérivé solvaté révèle que celui-ci est constitué de la simple superposition des deux premiers, ce qui confirme que le DMF ne constitue qu'un solvate.

L'examen des distances et angles de liaisons ne révèle pas de différences significatives entre le (thénoyl-2')-amino-2 nitro-5 thiazole diméthylformamide et la variété cristalline obtenue précédemment dans l'acétone.[1] La présence du DMF et tant que solvate n'entraîne donc pas de variations importantes au niveau de la géométrie moléculaire. Les différences significatives ne dépassent pas 0,04 Å pour les distances C(2')–C(3') et S(1')–C(2'), tandis que

TABLEAU II. Distances Interatomiques (en Å) et Angles (en Degrés)

Molécule			
S(1)–C(2)	1,721 (8)	C(4)–C(5)–S(1)	111,7 (6)
S(1)–C(5)	1,719 (7)	N(51)–C(5)–C(4)	126,4 (7)
C(2)–N(3)	1,321 (9)	N(51)–C(5)–S(1)	122,0 (6)
N(3)–C(4)	1,36 (1)	C(5)–N(51)–O(511)	119,5 (7)
C(4)–C(5)	1,35 (1)	O(511)–N(51)–O(512)	123,3 (7)
C(5)–N(51)	1,40 (1)	O(512)–N(51)–C(5)	117,2 (6)
N(51)–O(511)	1,235 (8)	S(1)–C(2)–N(21)	123,5 (5)
N(51)–O(512)	1,23 (1)	N(3)–C(2)–N(21)	119,9 (7)
C(2)–N(21)	1,36 (1)	C(2)–N(21)–H(N21)	113 (7)
N(21)–C(2'1)	1,374 (9)	C(2)–N(21)–C(2'1)	123,0 (7)
C(2'1)–O(2'11)	1,23 (1)	H(N21)–N(21)–C(2'1)	123 (7)
C(2'1)–C(2')	1,47 (1)	N(21)–C(2'1)–C(2')	116,9 (7)
C(2')–C(3')	1,37 (1)	N(21)–C(2'1)–O(2'11)	120,7 (7)
C(3')–C(4')	1,40 (1)	O(2'11)–C(2'1)–C(2')	122,4 (6)
C(4')–C(5')	1,35 (1)	C(2'1)–C(2')–S(1')	117,6 (6)
C(5')–S(1')	1,70 (1)	C(2'1)–C(2')–C(3')	130,8 (6)
S(1')–C(2')	1,726 (7)	S(1')–C(2')–C(3')	111,6 (5)
N(21)–H(N21)	0,68 (10)	C(2')–C(3')–C(4')	111,9 (7)
C(5)–S(1)–C(2)	87,2 (4)	C(3')–C(4')–C(5')	113,1 (8)
S(1)–C(2)–N(3)	116,5 (6)	C(4')–C(5')–S(1')	112,4 (7)
C(2)–N(3)–C(4)	109,2 (6)	C(5')–S(1')–C(2')	91,1 (4)
N(3)–C(4)–C(5)	115,3 (6)		
Solvate			
O(11)–C(12)	1,222 (9)	O(11)–C(12)–H(12)	125 (6)
C(12)–N(13)	1,32 (1)	H(12)–C(12)–N(13)	111 (6)
N(13)–C(14)	1,45 (1)	C(12)–N(13)–C(14)	121,9 (7)
N(13)–C(15)	1,44 (1)	C(12)–N(13)–C(15)	120,2 (7)
O(11)–C(12)–N(13)	124,1 (8)	C(14)–N(13)–C(15)	117,5 (8)

TABLEAU III. Equations des Différents Plans Moyens (Pondérés à Partir des Écarts-Type sur des Positions Atomiques), Distances de ces Atomes à ces Plans et Angle Dièdre entre les Plans

Paramètres rapportés à un système de coordonnées cartésiennes en Å

	l	m	n	p
Cycle A	6,1230	6,3394	1,2975	8,3157
Cycle B	5,4281	6,4322	3,3778	8,9173
DMF	5,8977	6,7103	−1,4707	7,1304

Distances en Å des atomes aux plans moyens (les écarts-type sont mis entre parenthèses)

S(1)	0,000 (2)	S(1')	0,000 (2)
C(2)	0,001 (7)	C(2')	−0,004 (7)
N(3)	0,002 (6)	C(3')	0,002 (8)
C(4)	−0,008 (8)	C(4')	0,002 (9)
C(5)	0,007 (8)	C(5')	−0,005 (9)
N(21)[a]	0,003	C(2'1)[a]	0,000
O(511)[a]	0,073		
N(51)[a]	0,067	O(2'11)[a]	0,118
O(512)[a]	0,120	N(21)[a]	−0,178

Angle dièdre: 7,1 ° entre plans A et B

Molécule de DMF

O(11)	−0,012 (6)	C(14)	−0,06 (1)
C(12)	0,016 (9)	C(15)	−0,02 (1)
N(13)	0,028 (6)	H(12)[a]	0,004

Plans moyens $lX+mY+nZ=p$. *a*) Atomes exclus du calcul du plan moyen.

les angles ne diffèrent pas de plus de 2° au niveau de C(2')–C(3')–C(4'), C(5)–N(51)–O(511) et C(4)–C(5)–S(1). Les deux cycles aminothiazole (cycle A) et thénoyle (cycle B) restent proches de la planéïté; les équations de leurs plans moyens ont été calculées (Tableau III), les atomes les plus éloignés C(4) pour le cycle A et C(5') pour le cycle B restent à une distance inférieure à 0,008 Å du plan moyen correspondant. L'atome d'azote N(21) est situé dans le plan de l'aminothiazole. L'angle dièdre formé par les plans moyens des cycles A et B est très nettement inférieur à celui trouvé dans la variété monoclinique (I) obtenue en absence de DMF.

La géométrie de la molécule de DMF est très proche de celle rencontrée dans le complexe moléculaire qu'elle forme avec le diacétoxy-1,7 trinitro-2,4,6 triazaheptane-2,4,6.[7] Par contre, ces valeurs diffèrent nettement de celles observées dans le complexe formé entre l'alpha-cyclodextrine et le DMF et dans lequel ce dernier est situé à l'intérieur de la cavité hôte formée par le macrocycle.[8] Dans ce cas, l'agitation thermique des atomes est très importante; celle-ci peut expliquer la longueur très faible de la liaison CO (1,10 (2) Å) ainsi que la grande valeur de l'un des angles autour de l'azote (133 (1)°). La molécule de DMF est quasi plane (Tableau III), les distances des atomes au plan moyen n'excédant pas 0,06 (1) Å; ces valeurs sont cependant nettement supérieures à celles rencontrées dans les deux composés cités[7,8] dans lesquels les distances restent inférieures à 0,01 Å.

La molécule de DMF est reliée à la molécule citée en titre par une liaison hydrogène mettant en jeu les atomes O(11) du DMF et N(21) du pont amidique N(21)–H(N21)···O(11) 2,733 (9) Å, 171 (10)°.

La Fig. 1 représentant la projection de la maille parallèlement à l'axe [010] indique l'absence de recouvrement entre les différentes molécules, à la différence de ce qui a été décrit dans le premier composé.[1]

TABLEAU IV. Principaux Contacts de van der Waals (Å)

C(2)–N(3^{i})	3,27 (1)	C(5)–C(12^{i})	3,68 (1)
C(2)–O(11)	3,47 (1)	N(51)–O(11^{i})	3,067 (9)
C(2)–C(4^{i})	3,48 (1)	N(51)–O(511^{iii})	3,077 (9)
C(2)–C(3′^{ii})	3,51 (1)	N(51)–N(51^{iii})	3,36 (1)
C(2)–C(2^{i})	3,69 (2)	N(51)–C(12^{i})	3,57 (1)
N(3)–O(11)	3,333 (9)	O(511)–O(512^{iii})	3,196 (8)
N(3)–C(12)	3,35 (1)	O(511)–O(511^{iii})	3,27 (1)
N(3)–N(3^{i})	3,37 (1)	O(511)–C(3′^{i})	3,30 (1)
N(3)–C(3′^{ii})	3,46 (1)	O(511)–C(14^{iv})	3,36 (1)
N(3)–C(4′^{ii})	3,52 (1)	O(511)–O(11^{i})	3,432 (8)
N(3)–C(2′^{ii})	3,60 (1)	O(511)–C(4′^{v})	3,68 (1)
N(3)–N(21^{i})	3,64 (1)	O(512)–O(11^{i})	3,222 (9)
N(3)–C(5′^{ii})	3,69 (1)	O(512)–C(14^{vi})	3,36 (1)
C(4)–N(21^{i})	3,32 (1)	O(512)–C(12^{i})	3,38 (1)
C(4)–C(4′^{ii})	3,46 (1)	O(512)–C(15^{vi})	3,38 (1)
C(4)–C(5′^{ii})	3,64 (1)	O(512)–N(13^{i})	3,554 (8)
C(5)–O(11^{i})	3,30 (1)	O(512)–C(4′^{v})	3,57 (1)
C(5)–O(511^{iii})	3,57 (1)	O(512)–C(15^{i})	3,62 (1)
N(21)–C(12)	3,55 (1)		
N(21)–N(21^{ii})	3,59 (1)		
N(21)–O(11^{ii})	3,62 (1)		
C(2′1)–O(11^{ii})	3,34 (1)		
C(2′1)–C(12^{ii})	3,45 (1)		
C(2′1)–O(11)	3,697 (9)		
O(2′1)–C(5′^{vii})	3,14 (1)		
O(2′1)–C(12^{ii})	3,26 (1)		
O(2′1)–N(13^{ii})	3,348 (9)		
O(2′1)–O(11^{ii})	3,363 (8)		
O(2′1)–S(1′^{vii})	3,381 (5)		
O(2′1)–C(15^{ii})	3,56 (1)		
S(1′)–C(12^{ii})	3,658 (9)		
C(2′)–C(12^{ii})	3,70 (1)		
C(3′)–O(11)	3,118 (9)		

Liaison hydrogène: N(21)–H(N21)···O(11) 2,733 (9) Å

Code de symétrie, i: $2-x, 1-y, 1-z$. ii: $2-x, -y, 1-z$. iii: $1-x, 1-y, 1-z$. iv: $3/2-x, 1/2+y, 1/2-z$. v: $-1+x, 1+y, z$. vi: $-1/2+x, 1/2-y, 1/2+z$. vii: $5/2-x, 1/2+y, 3/2-z$.

La cohésion cristalline est assurée par de nombreux contacts de van der Waals (Tableau IV) dont les plus courtes correspondent à N(51)–O(11^{i}): 3,067 (9) et N(51)–O(511^{iii}): 3,077 (9) Å. (Code de symétrie: i: $2-x, 1-y, 1-z$ et iii: $1-x, 1-y, 1-z$). Dans le Tableau V sont décrits les paramètres U_{ij} d'agitation thermique anisotrope.

Conclusion

Le (thénoyl-2′)-amino-2 nitro-5 thiazole utilisable par voie buccale est extrêmement bien toléré aux doses habituelles. Toutefois, quelques légers troubles digestifs ont pu être observés dans de très rares cas. Il a été envisagé qu'une forme cristalline différente de la variété monoclinique étudiée précédemment[1)] pouvait être mise en cause. L'étude biologique de la nouvelle variété solvatée par le DMF que nous venons de décrire permettra de vérifier cette hypothèse.

TABLEAU V. Paramètres d'Agitation Thermique Anisotrope ($\times 10^3$)

	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
S(1)	53 (1)	44 (1)	33 (1)	1,4 (9)	17 (9)	4 (1)
C(2)	41 (4)	34 (4)	36 (4)	−5 (4)	13 (4)	−1 (4)
N(3)	46 (4)	44 (4)	28 (3)	−3 (3)	8 (3)	0 (4)
C(4)	40 (5)	36 (5)	35 (4)	4 (4)	6 (4)	−3 (4)
C(5)	44 (4)	38 (5)	40 (4)	−1 (4)	16 (4)	0 (4)
N(51)	45 (4)	45 (5)	57 (4)	5 (4)	20 (4)	−1 (4)
O(511)	59 (4)	59 (4)	61 (4)	5 (3)	12 (3)	15 (3)
O(512)	65 (4)	66 (4)	54 (4)	8 (3)	31 (3)	15 (3)
N(21)	52 (4)	42 (4)	29 (4)	0 (3)	14 (3)	0 (4)
C(2'1)	48 (5)	39 (5)	37 (4)	−3 (4)	9 (4)	−12 (4)
O(2'11)	60 (4)	64 (4)	29 (3)	1 (3)	16 (3)	4 (3)
S(1')	53 (1)	69 (2)	40 (1)	16 (1)	8 (1)	−1 (1)
C(2')	41 (4)	39 (5)	27 (4)	3 (4)	3 (3)	−4 (4)
C(3')	43 (5)	38 (5)	43 (4)	3 (4)	14 (4)	5 (4)
C(4')	42 (5)	57 (6)	58 (5)	−4 (5)	16 (4)	3 (5)
C(5')	51 (5)	56 (6)	64 (6)	23 (5)	7 (5)	3 (5)
O(11)	56 (4)	73 (4)	27 (3)	−2 (3)	8 (3)	9 (3)
C(12)	40 (5)	51 (6)	44 (5)	−1 (4)	16 (4)	0 (4)
N(13)	41 (4)	52 (4)	28 (3)	3 (3)	9 (3)	4 (3)
C(14)	62 (7)	80 (8)	49 (6)	−3 (5)	14 (5)	18 (6)
C(15)	77 (8)	98 (9)	48 (6)	18 (6)	27 (5)	32 (7)

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Studies on Hypolipidemic Agents. III. Synthesis and Esterase-Inhibitory Activity of ω-Cycloalkyl-2-oxoalkyl Arenesulfonates

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Various ω-cycloalkyl-2-oxoalkyl arenesulfonates were synthesized and evaluated for esterase- and chymotrypsin-inhibitory activities and hypolipidemic activity. Among the tested compounds, 2-oxoalkyl arenesulfonates (**4**, **8** and **13**) having a cyclohexyl substituent at the terminus of the alkyl chain exhibited considerable esterase-inhibitory activity, and several compounds among **4** and **8** also exhibited potent hypolipidemic action. The structure–activity relationships of these compounds are discussed.

Keywords——alicyclic alkanoic acid; ω-cycloalkyl-2-oxoalkyl arenesulfonate; ω-oxyacycloalkyl-2-oxoalkyl arenesulfonate; α-diazoketone; α-hydroxyketone; esterase-inhibitory activity; chymotrypsin-inhibitory activity; hypolipidemic activity; structure–activity relationship

Treatment of hyperlipemia is currently considered to be important for the primary prevention of arteriosclerosis,[1,2] and drugs such as Lipocline,[3] Probucol[4] and their analogues have been clinically applied as hypolipidemic agents for this purpose. We have reported[5] that various 2-oxoalkyl arenesulfonates exhibited a selective esterase-inhibitory activity as well as a hypolipidemic effect, for which we postulated a novel action mechanism based on a decrease of uptake of triglycerides and cholesterol esters into the small intestinal mucosa owing to inhibition of the enzymes in the small intestinal lumen. This paper deals with syntheses, biological activities and structure–activity relationships of 2-oxoalkyl arenesulfonates having various cycloalkyl substituents on the oxoalkyl portion.

Synthesis

Two methods were applied to synthesize the 2-oxoalkyl arenesulfonates (**4**, **8** and **13**). The first one involves α-diazoketones (**2**, **7** and **12**) as intermediates (method A). The other involves α-hydroxyketones (**3** and **9**) as intermediates (method B). Some commercially unavailable cyclohexylalkanoic acids (**6**) were prepared by catalytic hydrogenation of the corresponding phenylalkanoic acids (**5**) in the presence of PtO_2 as a catalyst according to the procedure of Allinger and Freiberg.[6] Among the prepared carboxylic acids, **6d** and **6e** were produced as stereoisomeric mixtures of *trans* and *cis* isomers, and their ratio was estimated to be about 1:3 on the basis of the nuclear magnetic resonance (NMR) spectra. Because separation of the stereoisomers of **6d** and **6e** was difficult, they were used without separation in order to tentatively evaluate the activities. The intermediates, α-diazoketones (**2** and **7**), were prepared from the corresponding acyl halides by treatment with diazomethane, and the other intermediates, α-hydroxyketones (**3** and **9**), were prepared by chlorination of the diazoketones with hydrogen chloride followed by treatment with ethyl formate in methanolic potassium hydroxide according to the procedure of Levine and Walti.[7] Physical and spectral

TABLE I. Physical Data for **2**

$X\text{-}(CH_2)_nCOCHN_2$
2

Compd.[a] No.	X	n	MS (M^+)	^{1}H-NMR ($CDCl_3$) δ ppm
2a[b]		0	110	0.75—1.50 (4H, m), 2.00—2.35 (1H, br), 5.24 (1H, s)
2b		1	152	0.85—2.00 (9H, m), 2.30 (2H, s), 5.20 (1H, s)
2c[c]		0	152	1.00—2.00 (10H, m), 2.00—2.40 (1H, br), 5.22 (1H, s)
2d		1	166	0.70—2.10 (11H, m), 2.20 (2H, d, J=7.5 Hz), 5.21 (1H, s)
2e		2	180	0.60—2.00 (13H, m), 2.31 (2H, t, J=8 Hz), 5.23 (1H, s)
2j		3	194	0.60—2.10 (15H, m), 2.30 (2H, t, J=7.5 Hz), 5.20 (1H, s)
2g		0	140	1.70—2.60 (4H, m), 3.70—4.15 (2H, m), 4.20—4.60 (1H, m), 5.76 (1H, s)
2h		0	154	1.50—2.10 (4H, m), 2.20—2.70 (1H, m), 3.15—3.65 (2H, m), 3.70—4.20 (2H, m), 5.30 (1H, s)

a) All compounds were light yellowish oils, and yields of all compounds were nearly quantitative. *b*) Ref. 8. *c*) Ref. 9.

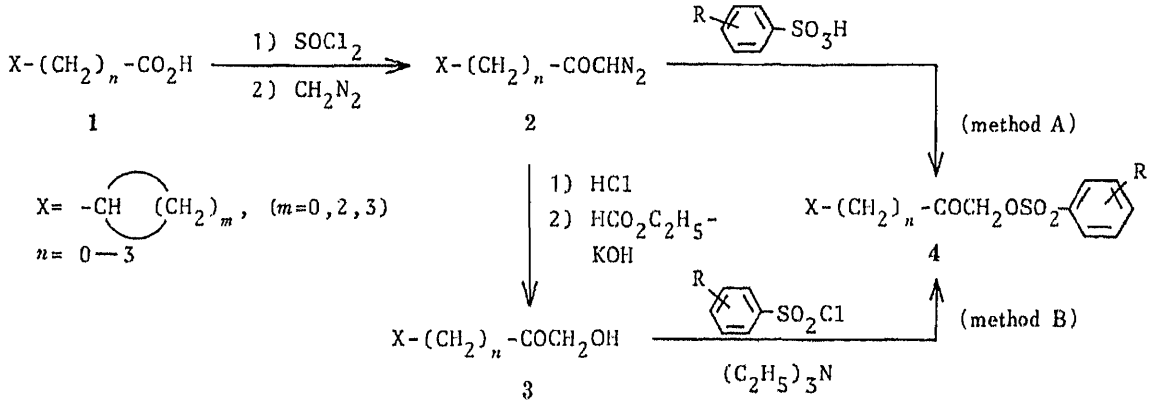


Chart 1

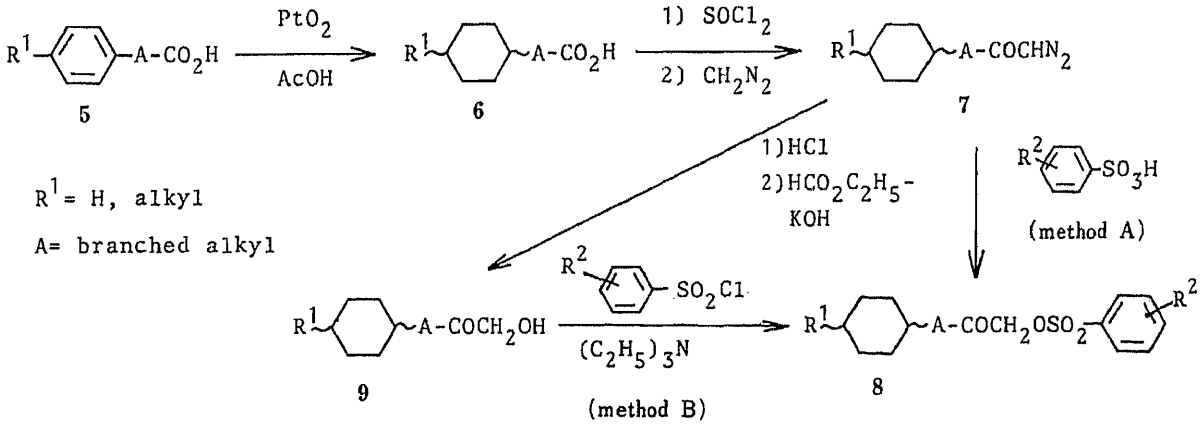
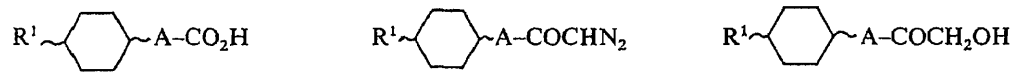


Chart 2

TABLE II. Physical Data for **3**, **6**, **7**, and **9**



6 **7** **3** and **9**

Compd.[a] No.	R^1	A	bp °C/mmHg (mp °C)	MS (M^+)	^{1}H-NMR ($CDCl_3$) δ ppm
6a[b]	H	$-CH_2\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-$	129/1	170	0.70—1.90 (13H, m), 1.18 (3H, d, J=7 Hz), 2.35—2.70 (1H, m), 11.22 (1H, br)
6b	H	$-CH_2\underset{\displaystyle C_2H_5}{\underset{\vert}{CH}}-$	135—136/1	184	0.96 (3H, t, J=7.5 Hz), 0.70—2.15 (15H, m), 2.20—2.65 (1H, m), 10.75 (1H, br)
6c	H	$-CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}-$	(68—69)	184	0.80—1.85 (13H, m), 1.19 (6H, s), 10.00 (1H, br)
6d[c]	$-C_2H_5$	—	120/1	156	0.89 (3H, t, J=7.5 Hz), 0.70—2.15 (11H, m), 2.15—2.65 (1H, m), 11.55 (1H, br)
6e[d]	$-CH(CH_3)_2$	—	131—134/1	170	0.88 (6H, d, J=8 Hz), 0.95—2.20 (10H, m), 2.20—2.70 (1H, m), 11.35 (1H, br)
7a	H	$-CH_2\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-$	Oil	194	0.65—1.85 (16H, m), 2.25—2.63 (1H, m), 5.22 (1H, s)
7b	H	$-CH_2\underset{\displaystyle C_2H_5}{\underset{\vert}{CH}}-$	Oil	208	0.65—2.00 (18H, m), 2.10—2.50 (1H, m), 5.20 (1H, s)
7c	H	$-CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}-$	Oil	208	0.75—1.85 (13H, m), 1.12 (6H, s), 5.42 (1H, s)
7d	$-C_2H_5$	—	Oil	180	0.70—2.20 (14H, m), 2.20—2.43 (1H, s), 5.21, 5.28 (1H, s)
7e	$-CH(CH_3)_2$	—	Oil	194	0.86 (6H, s), 0.80—2.05 (10H, m), 2.05—2.50 (1H, m), 5.22, 5.30 (1H, s)
3a[e]	H	—	115—116/4	142	1.00—2.00 (10H, m), 2.10—2.55 (1H, m), 3.14 (1H, t, J=4 Hz), 4.26 (2H, d, J=4 Hz)
3b	H	$-CH_2CH_2-$	95—97/2	170	0.60—2.00 (13H, m), 2.40 (2H, t, J=8 Hz), 3.14 (1H, t, J=4 Hz), 4.20 (2H, d, J=4 Hz)
9a	H	$-CH_2\underset{\displaystyle C_2H_5}{\underset{\vert}{CH}}-$	110/1	198	0.89 (3H, t, J=7.5 Hz), 0.70—2.00 (15H, m), 2.30—2.68 (1H, m), 3.18 (1H, br), 4.20 (2H, s)
9b	H	$-CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}-$	Oil[f]	198	0.70—1.80 (13H, m), 1.16 (6H, s), 3.26 (1H, t, J=4 Hz), 4.38 (2H, d, J=4 Hz)

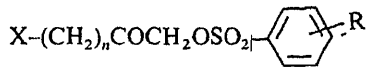
a) Compounds **6d**, **6e**, **7d** and **7e** are stereoisomeric mixtures. *b*) Ref. 10. *c*) Ref. 11. *d*) Ref. 12. *e*) Ref. 13. *f*) Purified by column chromatography on silica gel. Not distilled.

data for the obtained cyclohexylalkanoic acids (**6**), α-diazoketones (**2** and **7**) and α-hydroxyketones (**3** and **9**) are listed in Tables I and II.

The diazoketones (**2** and **7**) were converted to the corresponding arenesulfonates (**4a**—**u** and **8a**—**h**) in good yields by treatment with arenesulfonic acids according to the procedure of Crowther and Holt[14] (method A). Otherwise, the α-hydroxyketones (**3a**, **b** and **9a**, **b**) were esterified with various arenesulfonyl chlorides in the presence of triethylamine to afford the corresponding arenesulfonates (**4d**, **l**, **m** and **8d**, **f**) (method B).

3-Cyclohexyloxy-2-oxoalkyl arenesulfonates (**13**) were similarly obtained by method A starting from the corresponding carboxylic acids (**11**), which were prepared by catalytic hydrogenation of the corresponding 2-phenoxyalkanoic acids (**10**) in the presence of the Rh–Pt (3 : 1) as a catalyst. Physical, spectral, and biological data for the obtained 2-oxoalkyl

TABLE III. Enzyme-Inhibitory Activities of **4**



4

Compd. No.	X	*n*	R	Method[a]	Yield[b] (%)	mp (°C)	Inhibitions: Esterase[d] IC_{50} (μM)	Inhibitions: Chymotry.[e] (1×10^{-4} M)	Reduction[f]: Trigly.[g]
4a		0	4-OCH_3	A	37	Oil[c]	>1000	3	—[h]
4b		0	2,4,6-$(CH_3)_3$	A	41	82—83 (Et–W)[i]	>1000	14	—[h]
4c		1	4-CH_3	A	65	51—52 (M–W)	4.4	90	—[h]
4d		0	H	A B	61 67	Oil[c]	1.6	13	—[h]
4e		0	4-Cl	A	55	69—70 (PE–E)	1.5	32	—[h]
4f		0	4-OH	A	67	124—125 (M–W)	2.6	15	—[h]
4g		0	2,4,6-$(CH_3)_3$	A	68	95—96 (M–W)	0.9	10	—[h]
4h		1	4-CH_3	A	52	32—33 (PE–E)	3.5	98	—[h]
4i		1	4-OH	A	60	92—93 (Et–W)	0.8	—[h]	—[h]
4j		1	4-OC_2H_5	A	67	48—49 (PE)	5.9	97	55
4k		1	2,4,6-$(CH_3)_3$	A	68	37—38 (PE–E)	0.2	10	70
4l		2	H	A B	66 73	46—47 (M–W)	2.2	100	77
4m		2	4-CH_3	A B	69 77	73—74 (M–W)	3.1	100	—[h]
4n		2	4-OC_2H_5	A	54	44—45 (PE–E)	4.0	94	82
4o		2	4-NO_2	A	83	77—78 (PE–E)	7.4	98	—[h]
4p		3	4-OC_2H_5	A	72	40—41 (PE)	6.5	98	56
4q		0	4-CH_3	A	25	103—106 (M–W)	>1000	0	—[h]
4r		0	H	A	60	54—55 (E)	220	9	—[h]
4s		0	4-OH	A	51	154—155 (Et–W)	240	—[h]	—[h]

TABLE III. (continued)

Compd. No.	X	n	R	Method[a]	Yield[b] (%)	mp (°C)	Inhibitions: Esterase[d] IC_{50} (μM)	Inhibitions: Chymotry.[e] (1×10^{-4} M)	Reduction[f] Trigly.[g]
4t		0	4-OC_2H_5	A	55	57—58 (Et–W)	350	—[h]	—[h]
4u		0	2,4,6-$(CH_3)_3$	A	64	64—65 (PE)	32	—[h]	—[h]

a) See the experimental section. *b*) Yield from the corresponding diazoketone (**2**) (method A) or α-ketoalcohol (**3**) (method B). *c*) Purified by column chromatography on silica gel. *d*) Methyl butyrate was used as a substrate. *e*) ATEE was used as a substrate. Expressed as percentage inhibition of chymotrypsin inhibitory activity at 1×10^{-4} M. *f*) Expressed as percentage deviation from the control value. Dose: 0.3 mmol/kg *p.o.* in rats. See the experimental section. *g*) Plasma triglyceride. *h*) Not tested. *i*) Recrystallization solvents: Et = ethanol, M = methanol, E = ethyl ether, PE = petroleum ether W = H_2O.

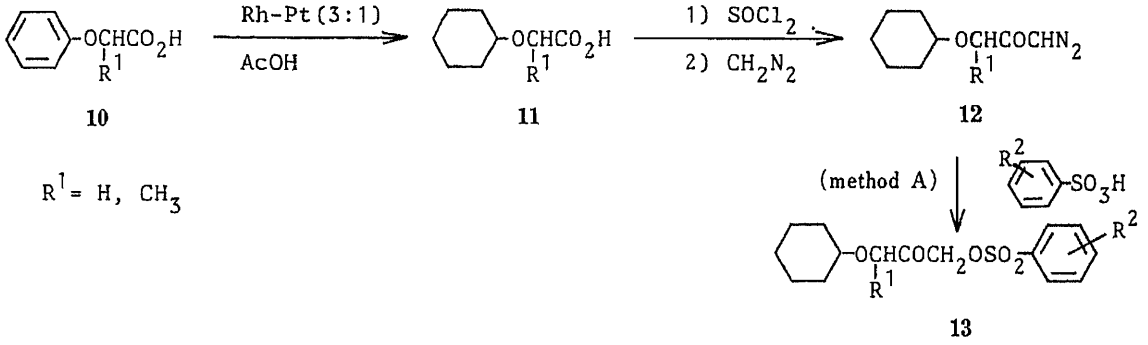


Chart 3

arenesulfonates (**4**, **8** and **13**) are listed in Tables III—VII.

Enzyme-Inhibitory Activity (*in Vitro* Experiments)

Methyl butyrate and *N*-acetyltyrosine ethyl ester (ATEE) were used as substrates for the activity determination of esterase[15] and chymotrypsin,[15] respectively (Tables III, V and VI).

Pharmacological Examination (*in Vivo* Experiment)

Male Wistar rats (7 weeks old) were used, with five animals in each experimental group. A test compound (0.3 mmol) was mixed with 5 ml of olive oil and the mixture was orally administered to rats at the dose of 0.3 mmol/kg. A blood sample for the determination of plasma triglyceride was taken from the orbital vein of the rats at 2 h after the administration. Plasma triglyceride was analyzed by using a commercially available analysis kit (Determiner TG-S Kyowa[16]). Decrease of triglyceride was expressed as the percentage deviation from the control value obtained by using olive oil containing no test compound.

Results and Discussion

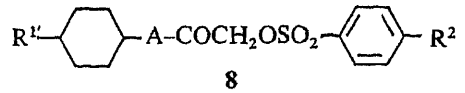
On the basis of the biological data from the *in vitro* and *in vivo* screening tests, the structure–activity relationships of the arenesulfonates may be summarized as follows. i) The biological data for the substituted arenesulfonates (**4**), which have various cycloalkyl or oxacycloalkyl substituents with various methylene chain lengths (n=0—3), are listed in Table III. The data indicate that the cyclopentyl (only one example, **4c**) and cyclohexyl

TABLE IV. Physical Data for **4**

Compd. No.	Formula	Analysis (%) Calcd (Found) C	H	N	^{1}H-NMR ($CDCl_3$) δ ppm
4a	$C_{12}H_{14}O_5S$	53.32 (53.37	5.22 5.13)		0.88—1.30 (4H, m), 1.90—2.30 (1H, m), 3.82 (3H, s), 4.60 (2H, s), 7.00 (2H, d, J=9 Hz), 7.84 (2H, d, J=9 Hz)
4b	$C_{14}H_{18}O_4S$	59.50 (59.57	6.43 6.73)		0.90—1.20 (4H, m), 2.00—2.40 (1H, m), 2.30 (3H, s), 2.65 (6H, s), 4.55 (2H, s), 7.00 (2H, s)
4c	$C_{15}H_{20}O_4S$	60.78 (60.71	6.80 6.90)		0.80—2.30 (9H, m), 2.43 (3H, s), 2.48 (2H, d, J=5 Hz), 4.46 (2H, s), 7.36 (2H, d, J=8 Hz), 7.84 (2H, d, J=8 Hz)
4d	$C_{14}H_{18}O_4S$	59.50 (59.65	6.43 6.27)		1.00—2.00 (10H, m), 2.00—2.70 (1H, br), 4.61 (2H, s), 7.44—8.05 (5H, m)
4e	$C_{14}H_{17}ClO_4S$	53.07 (53.04	5.40 5.44)		1.00—2.00 (10H, m), 2.20—2.65 (1H, m), 4.62 (2H, s), 7.50 (2H, d, J=8 Hz), 7.84 (2H, d, J=8 Hz)
4f	$C_{14}H_{18}O_5S$	56.36 (56.34	6.08 6.11)		1.00—1.90 (10H, m), 2.18—2.56 (1H, br), 3.28 (1H, s), 4.82 (2H, s), 6.90 (2H, d, J=9 Hz), 7.73 (2H, d, J=9 Hz)
4g	$C_{17}H_{24}O_4S$	63.24 (63.02	7.45 7.60)		1.00—2.00 (11H, m), 2.28 (3H, s), 2.60 (6H, s), 4.46 (2H, s), 6.98 (2H, s)
4h	$C_{16}H_{22}O_4S$	61.91 (61.93	7.14 7.13)		0.70—2.00 (11H, m), 2.29 (2H, d, J=6 Hz), 2.43 (3H, s), 4.44 (2H, s), 7.34 (2H, d, J=8 Hz), 7.82 (2H, d, J=8 Hz)
4i	$C_{15}H_{20}O_5S$	57.67 (57.75	6.45 6.53)		0.70—2.00 (11H, m), 2.33 (2H, d, J=6 Hz), 4.53 (2H, s), 6.93 (2H, d, J=9 Hz), 7.77 (2H, d, J=9 Hz)
4j	$C_{17}H_{24}O_5S$	59.97 (59.93	7.10 6.90)		0.70—2.15 (14H, m), 2.34 (2H, d, J=6 Hz), 4.10 (2H, q, J=6.5 Hz), 4.45 (2H, s), 6.98 (2H, d, J=8.5 Hz), 7.81 (2H, d, J=8.5 Hz)
4k	$C_{18}H_{26}O_4S$	63.88 (63.86	7.74 7.48)		0.80—2.00 (11H, m), 2.30 (3H, s), 2.34 (2H, d, J=6.5 Hz), 2.65 (6H, s), 4.40 (2H, s), 7.00 (2H, s)
4l	$C_{16}H_{22}O_4S$	61.90 (61.74	7.14 7.29)		0.60—1.85 (13H, m), 2.45 (2H, t, J=7 Hz), 4.50 (2H, s), 7.35—8.00 (5H, m)
4m	$C_{17}H_{24}O_4S$	62.93 (62.89	7.45 7.25)		0.80—2.30 (13H, m), 2.42 (2H, t, J=7 Hz), 2.46 (3H, s), 4.45 (2H, s), 7.34 (2H, d, J=8 Hz), 7.83 (2H, d, J=8 Hz)
4n	$C_{18}H_{26}O_5S$	60.99 (60.90	7.39 7.56)		0.60—1.85 (13H, m), 1.44 (3H, t, J=7 Hz), 2.44 (2H, t, J=7 Hz), 4.08 (2H, q, J=7 Hz), 4.45 (2H, s), 7.00 (2H, d, J=9 Hz), 7.85 (2H, d, J=9 Hz)
4o	$C_{16}H_{21}NO_6S$	54.07 (54.04	5.96 6.01	3.94 3.74)	0.70—1.85 (13H, m), 2.44 (2H, t, J=7.5 Hz), 4.66 (2H, s), 8.02 (2H, d, J=9 Hz), 8.32 (2H, d, J=9 Hz)
4p	$C_{19}H_{28}O_5S$	61.93 (61.94	7.65 7.62)		0.60—1.85 (18H, m), 2.44 (2H, t, J=7 Hz), 4.10 (2H, q, J=6.5 Hz), 4.46 (2H, s), 6.98 (2H, d, J=8.5 Hz), 7.81 (2H, d, J=8.5 Hz)
4q	$C_{13}H_{16}O_5S$	54.92 (55.03	5.67 6.00)		1.60—2.25 (4H, m), 2.42 (3H, m), 3.10—3.40 (2H, m), 4.03 (2H, q, J=18 Hz), 5.35—5.60 (1H, m), 7.32 (2H, d, J=8.5 Hz), 7.82 (2H, d, J=8.5 Hz)
4r	$C_{13}H_{16}O_5S$	54.92 (54.92	5.67 5.76)		1.50—1.80 (4H, m), 2.60—3.00 (1H, m), 3.20—3.60 (2H, m), 3.80—4.15 (2H, m), 4.62 (2H, s), 7.40—8.10 (5H, m)
4s	$C_{13}H_{16}O_6S$	51.99 (51.86	5.37 5.51)		1.50—1.90 (4H, m), 2.50—3.00 (1H, m), 3.20—3.60 (2H, m), 3.80—4.10 (2H, m), 4.54 (2H, s), 6.96 (2H, d, J=9 Hz), 7.72 (2H, d, J=9 Hz)
4t	$C_{15}H_{20}O_6S$	54.86 (54.57	6.13 6.24)		1.43 (3H, t, J=8 Hz), 1.50—2.00 (4H, m), 2.60—3.00 (1H, m), 3.20—3.55 (2H, m), 3.80—4.10 (2H, m), 4.06 (2H, q, J=8 Hz), 4.53 (2H, s), 7.00 (2H, d, J=9 Hz), 7.84 (2H, d, J=9 Hz)
4u	$C_{16}H_{22}O_5S$	58.87 (58.84	6.79 6.91)		1.50—1.85 (4H, m), 2.28 (3H, s), 2.60 (6H, s), 2.50—3.00 (1H, m), 3.20—3.60 (2H, m), 3.80—4.10 (2H, m), 4.50 (2H, s), 6.98 (2H, s)

derivatives (**4h, j, l—p**) show potent esterase- and chymotrypsin-inhibitory activities, but the cyclopropyl (**4a, b**) and oxacycloalkyl derivatives (**4q—u**) are not effective. On the other hand, the arenesulfonates, **4d—g** and **4k** with n=0, show only esterase inhibition. The value of n and the substituent on the phenyl group have no significant effect on the esterase-inhibitory

TABLE V. Enzyme-Inhibitory Activities and Hypolipidemic Effect of **8**

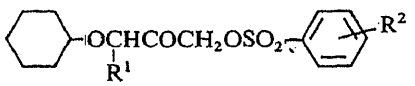


8

Compd. No.	R^1	A	R^2	Method[a]	Yield[b] (%)	mp (°C)	Inhibitions: Esterase[d] IC_{50} (μM)	Inhibitions: Chymotry.[e] (1×10^{-4} M)	Reduction[f]: Trigly.[g]
8a	H	$-CH_2CH(CH_3)-$	H	A	75	51—52 (Et–W)[i]	0.35	31	60
8b	H	$-CH_2CH(CH_3)-$	$-CH_3$	A	71	50—51 (E)	7.8	31	50
8c	H	$-CH_2CH(CH_3)-$	$-OCH_3$	A	78	33—34 (PE–E)	4.0	22	63
8d	H	$-CH_2CH(C_2H_5)-$	H	A B	70 82	Oil[c]	4.7	24	80
8e	H	$-CH_2CH(C_2H_5)-$	$-OCH_3$	A	73	Oil[c]	—[h]	—[h]	52
8f	H	$-CH_2C(CH_3)-$	H	A B	68 83	40—41 (PE–E)	—[h]	—[h]	—[h]
8g[j]	$-C_2H_5$	—	H	A	75	Oil[c]	0.2	58	—[h]
8h[j]	$-CH(CH_3)_2$	—	H	A	81	Oil[c]	0.4	23	87

a—*i*) See the corresponding footnotes in Table III. *j*) These compounds were stereoisomeric mixtures.

TABLE VI. Enzyme-Inhibitory Activities of [13]



13

Compd. No.	R^1	R^2	Yield[a] (%)	mp[b] (°C)	Inhibitions: Esterase[c] IC_{50} (μM)	Inhibitions: Chymotry.[d] (1×10^{-4} M)
13a	H	H	64.	Oil	18.0	52
13b	H	4-CH_3	73	Oil	7.8	51
13c	H	4-OCH_3	68	Oil	4.6	50
13d	CH_3	H	78	Oil	34.0	14
13e	CH_3	4-CH_3	70	Oil	29.0	8
13f	CH_3	2,4,6-$(CH_3)_3$	85	Oil	6.2	20

a) Yield from the corresponding diazoketone (**12**) (method A). *b*) Purified by column chromatography on silica gel. *c*) Methyl butyrate was used as a substrate. *d*) ATEE was used as a substrate. Expressed as percentage inhibition of chymotrypsin-inhibitory activity at 1×10^{-4} M.

activity, but the compounds with $n=1$, 2 and 3 in Table III are more effective as chymotrypsin inhibitors than the compounds with $n=0$. ii) The data in Table V indicate that alkyl substituents on the side chain or on the cyclohexane ring of the arenesulfonates (**8**) do not have any appreciable effect on the esterase inhibition, but tend to cause a considerable

TABLE VII. Physical Data for 8 and 13

Compd. No.	Formula	Analysis (%) Calcd (Found) C	H	^{1}H-NMR ($CDCl_3$) δ ppm
8a	$C_{17}H_{24}O_4S$	62.94 (63.04	7.46 7.55)	0.70—1.80 (16H, m), 2.60—2.90 (1H, m), 4.61 (2H, s), 7.40—8.00 (5H, m)
8b	$C_{18}H_{26}O_4S$	63.88 (63.94	7.74 7.91)	0.75—1.80 (16H, m), 2.44 (3H, s), 2.60—2.95 (1H, m), 4.59 (2H, s), 7.30 (2H, d, J=8.5 Hz), 7.78 (2H, d, J=8.5 Hz)
8c	$C_{18}H_{26}O_5S$	60.99 (60.85	7.39 7.62)	0.70—1.85 (16H, m), 2.60—2.92 (1H, m), 3.88 (3H, s), 4.56 (2H, s), 6.97 (2H, d, J=9 Hz), 7.81 (2H, d, J=9 Hz)
8d	$C_{18}H_{26}O_4S$	63.88 (63.52	7.74 7.86)	0.60—1.90 (18H, m), 2.40—2.80 (1H, m), 4.58 (2H, s), 7.35—8.05 (5H, m)
8e	$C_{19}H_{28}O_5S$	61.93 (62.33	7.66 7.72)	0.65—1.85 (18H, m), 2.42—2.82 (1H, m), 3.88 (3H, s), 4.53 (2H, s), 6.98 (2H, d, J=8.5 Hz), 7.84 (2H, d, J=8.5 Hz)
8f	$C_{18}H_{26}O_4S$	63.88 (63.99	7.74 7.89)	0.70—1.80 (13H, m), 1.10 (6H, s), 4.90 (2H, s), 7.40—7.80 (3H, m), 7.90—8.10 (2H, m)
8g	$C_{16}H_{22}O_4S$	61.91 (61.80	7.14 7.30)	0.60—2.00 (14H, m), 2.20—2.70 (1H, m), 4.62 (2H, s), 7.35—8.00 (5H, m)
8h	$C_{17}H_{24}O_4S$	62.93 (63.17	7.46 7.66)	0.70—2.10 (16H, m), 2.50—2.70 (1H, m), 4.61, 4.63 (2H, s), 7.35—8.00 (5H, m)
13a	$C_{15}H_{20}O_5S$	57.67 (57.38	6.45 6.75)	0.90—2.12 (10H, m), 3.10—3.45 (1H, br), 4.18 (3H, s), 4.91 (2H, s), 7.48—8.14 (5H, m)
13b	$C_{16}H_{22}O_5S$	58.88 (58.70	6.79 6.85)	0.90—2.08 (10H, m), 2.47 (3H, s), 3.05—3.44 (1H, br), 4.18 (2H, s), 4.86 (2H, s), 7.39 (2H, d, J=8 Hz), 7.86 (2H, d, J=8 Hz)
13c	$C_{16}H_{22}O_6S$	56.12 (56.33	6.48 6.41)	1.00—2.02 (10H, m), 3.10—3.40 (1H, br), 3.91 (3H, s), 4.18 (2H, s), 4.84 (2H, s), 7.05 (2H, d, J=9 Hz), 7.92 (2H, d, J=9 Hz)
13d	$C_{16}H_{22}O_5S$	58.88 (58.90	6.79 6.49)	0.85—2.08 (10H, m), 1.28 (3H, d, J=7 Hz), 3.10—3.48 (1H, br), 4.07 (1H, q, J=7 Hz), 5.02 (2H, s), 7.46—8.12 (5H, m)
13e	$C_{17}H_{24}O_5S$	59.98 (59.67	7.11 7.31)	0.90—2.10 (10H, m), 1.28 (3H, d, J=7 Hz), 2.46 (3H, s), 3.10—3.50 (1H, br), 4.07 (1H, q, J=7 Hz), 4.99 (2H, s), 7.37 (2H, d, J=8 Hz), 7.86 (2H, d, J=8 Hz)
13f	$C_{19}H_{28}O_5S$	61.93 (62.35	7.66 8.07)	0.90—2.08 (10H, m), 1.27 (3H, d, J=7 Hz), 2.32 (3H, s), 2.66 (6H, s), 3.10—3.54 (1H, br), 4.06 (1H, q, J=7 Hz), 4.95 (2H, s), 7.00 (2H, s)

decrease of the chymotrypsin-inhibitory activity in comparison with that of **4** (*i.e.* **4h, j, l—p**). iii) The data in Table VI indicate that a 3-cyclohexyloxy substituent (**13a—f**) has a moderate effect on both the esterase- and the chymotrypsin-inhibitory activities, which do not exceed the values for **4c, h, j, l—o** and **4p** as regards the chymotrypsin-inhibitory activity or those of **4e, g, i, k** and **8a, g, h** as regards the esterase-inhibitory activity. A branch (R^1 = CH_3) on the alkyl moiety decreases the chymotrypsin-inhibitory action. iv) Hypolipidemic evaluations of **4j—l, n, p** and **8a—h** were carried out (Tables III and V). Among the tested compounds, the arenesulfonates (**4k, l, n** and **8d, h**) afforded good results (70%, 77%, 82%, 80% and 87% reductions of the triglyceride in plasma, respectively), though the *in vitro* chymotrypsin-inhibitory activities of **4k, 8d** and **8h** (but not **4l** or **4n**) are considerably lower. This result indicates that the chymotrypsin-inhibitory action is not directly related to the reduction of the triglyceride in plasma.

Conclusion

We prepared a series of the ω-cycloalkyl-2-oxoalkyl arenesulfonate derivatives and related compounds in order to find effective hypolipidemic agents. The potencies of the esterase-inhibitory activity of the present arenesulfonates (**4, 8** and **13**) were somewhat lower

than those of the previously reported 2-oxoalkyl arenesulfonates[5b] with no cycloalkyl substituent, but in *in vivo* examinations of the series of the arenesulfonates, **4** and **8** showed more potent hypolipidemic action (**4n** and **8d**, **h**; 82%, 80% and 87% reductions of the triglyceride in plasma, respectively) than previously reported compounds. Our search for more favorable hypolipidemic agents is continuing on the basis of the present results. Unfortunately, in the present studies, the favored compounds (**8g**, **h**) were both tested as stereoisomeric mixtures, and separation of the stereoisomers would be desirable in order to investigate the biological activities in more detail. Recently, we have found a more potent hypolipidemic agent among the separated stereoisomers of analogues of **8g** and **8h**. Further investigations on the stereochemistry and structure–activity relationships of the arenesulfonates (**8g**, **h** and their analogues) will be reported in a forthcoming paper.

Experimental

All melting points were recorded with a Yanagimoto micromelting point apparatus and are uncorrected. Spectral data were obtained as follows: mass spectra (MS) with a JEOL 01G-2 spectrometer; proton nuclear magnetic resonance (^{1}H-NMR) spectra with a JEOL JMN-FX 100 spectrometer (using tetramethylsilane as an internal standard). Chemical shifts of ^{1}H-NMR spectra are given in δ values (ppm).

2-Cyclopropyl-1-diazo-2-ethanone (2a) (Typical Procedure)——A mixture of thionyl chloride (20 ml) and cyclopropanecarboxylic acid (**1a**) (1.5 g) was stirred for 5 h under reflux and then the reaction mixture was evaporated under reduced pressure. The residue (cyclopropionyl chloride) was added dropwise to an ethereal solution (100 ml) of diazomethane (obtained from 7.0 g of nitrosomethylurea) under stirring with ice-cooling. After being stirred for 1 h, the reaction mixture was evaporated to dryness under reduced pressure to give **2a**[8] quantitatively as a light yellowish oil. Other compounds (**2b**—**h**) were similarly prepared. Other data are listed in Table I.

4-Cyclohexyl-1-hydroxy-2-butanone (3b) (Typical Procedure)——Dry hydrogen chloride was passed into an ethereal solution (200 ml) of **2e** (12.0 g) until saturation under stirring with ice-cooling. After being stirred for 0.5 h, the reaction mixture was evaporated under reduced pressure to give 1-chloro-4-cyclohexyl-2-butanone, which was added to a solution of ethyl formate (6.4 g) and potassium hydroxide (4.9 g) in 80% aqueous ethanol (100 ml). After being refluxed for 4 h, the reaction mixture was evaporated under reduced pressure. The residue was extracted with chloroform (100 ml) and the organic layer was washed with water. The chloroform layer was dried over sodium sulfate and evaporated under reduced pressure to give **3b** as a crude oil, which was purified by distillation. Yield, 5.0 g (44%). 2-Cyclohexyl-1-hydroxy-2-ethanone (**3a**)[13] was similarly prepared from 2-cyclohexyl-1-diazo-2-ethanone (**2c**).[9] Yield, 4.0 g (48%). Other data are listed in Table II.

2-Cyclopropyl-2-oxoethyl 4-Methoxybenzenesulfonate (4a) (Typical Procedure)——Method A: The title compound (**4a**) was prepared from **2a** (1.1 g) and 4-methoxybenzenesulfonic acid (3.8 g) in the same manner as described in the previous paper.[5] Yield, 1.0 g (37%). Compounds **4b**—**u** were similarly prepared from the diazoketones (**2a**—**h**) and the corresponding arenesulfonic acids. Other data are listed in Tables III and IV.

2-Cyclohexyl-2-oxoethyl Benzenesulfonate (4d) (Typical Procedure)——Method B: Triethylamine (3.5 ml) was added dropwise to a stirred solution of benzenesulfonyl chloride (3.4 g) and **3a** (2.8 g) in dichloromethane (10 ml) at 0—5 °C. after being stirred for 3 h, the reaction mixture was extracted with chloroform (100 ml) and the organic layer was washed with 1 N HCl (20 ml × 2). The chloroform layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform as an eluent and the eluate was evaporated under reduced pressure to give an oily product, which was identical with **4d** obtained by method A in terms of the ^{1}H-NMR spectrum. Yield, 3.8 g (67%). Compounds **4l** and **4m** were similarly prepared from the α-hydroxyketone (**3b**) and arenesulfonyl chloride. Other data are listed in Table III.

3-Cyclohexyl-2-methylpropionic Acid (6a) (Typical Procedure)——A mixture of 2-methyl-3-phenylpropionic acid (**5a**)[17] (25.0 g) and PtO_2 (1.0 g) in acetic acid (150 ml) was hydrogenated under a pressure of 50 atm for 4 h at room temperature. The reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The oily residue was purified by distillation to give **6a**. Yield, 23.0 g (89%). bp 129 °C/1 mmHg (lit.,[10] bp 178—179 °C/2 mmHg). Compounds **6b**—**e** were similarly prepared from the corresponding arylcarboxylic acids (**5b**—**e**). Other data are listed in Table II.

3-Cyclohexylmethyl-1-diazo-2-butanone (7a) (Typical Procedure)——The title compound (**7a**) was prepared from **6a** (2.0 g) in the same manner as described for **2a**. Compounds **7b**—**e** were similarly prepared from the corresponding carboxylic acids (**6b**—**e**). Other data are listed in Table II.

3-Cyclohexylmethyl-1-hydroxy-2-pentanone (9a) (Typical Procedure)——The title compound (**9a**) was prepared from **7b** (2.0 g) in the same manner as described for **3b**. Yield, 1.4 g (74%). Compound **9b** was similarly prepared from the corresponding diazoketone (**7c**) (1.5 g). The crude product was purified by column chromatography on silica gel

with chloroform as an eluent to give **9b** as an oil. Yield, 0.8 g (56%). Other data are listed in Table II.

3-Cyclohexylmethyl-2-oxobutyl Benzenesulfonate (8a) (Typical Procedure)——Method A: The title compound (**8a**) was prepared from **7a** (2.0 g) and benzenesulfonic acid (3.5 g) in an ethereal solution (100 ml) in the same manner as described for **4a** (method A). Yield, 2.5 g (75%). Compounds **8b**—**h** were similarly prepared from the diazoketones (**7a**—**e**) and the corresponding arenesulfonic acids. Other data are listed in Tables V and VI.

3-Cyclohexylmethyl-2-oxopentyl Benzenesulfonate (8d) (Typical Procedure)——Method B: The title compound (**8d**) was prepared from **9a** (1.5 g) and benzenesulfonyl chloride (1.3 g) in dichloromethane (3 ml) in the same manner as described for **4d** (method B). The product was identical with **8d** obtained by method A, in terms of the ^{1}H-NMR spectrum. Yield, 2.1 g (82%). The compound (**8f**) was similarly prepared from the α-hydroxyketone (**9b**) and the corresponding arenesulfonyl chloride. Other data are listed in Table V.

Cyclohexyloxyacetic Acid (11a)——The title compound (**11a**) was prepared by hydrogenation of phenoxyacetic acid (**10a**) (25.0 g) in the presence of Rh–Pt (3 : 1, 1.5 g) as a catalyst in acetic acid (150 ml) under a pressure of 50 atm for 3 h at room temperature. The reaction mixture was worked-up in the same manner as described for **6a**. The oily residue was purified by distillation to give **11a**. Yield, 21.0 g (81%). bp 110—113 °C/2 mmHg (lit.,[18] bp 155—159 °C/20 mmHg). MS *m/z*: 158 (M^+). 2-Cyclohexyloxypropionic acid (**11b**) was similarly prepared from 2-phenoxypropionic acid (**10b**) (25.0 g). Yield, 22.0 g (85%). bp 120—123 °C/2 mmHg (lit.,[19] bp 117—119 °C/1.5 mmHg). MS *m/z*: 172 (M^+).

3-Cyclohexyloxy-1-diazo-2-propanone (12a) (Typical Procedure)——The title compound (**12a**) was quantitatively prepared starting from **11a** (3.0 g) in the same manner as described for **2a**. MS *m/z*: 182 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.80—2.15 (10H, m), 3.14—3.50 (1H, br), 4.04 (2H, s), 5.80 (1H, s). 3-Cyclohexyloxy-1-diazo-2-butanone (**12b**, oil). Yield, nearly quantitative. MS *m/z*: 196 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 0.90—2.15 (13H, m), 3.10—3.50 (1H, br), 4.02 (1H, q, J=7 Hz), 5.80 (1H, s).

3-Cyclohexyloxy-2-oxopropyl Benzenesulfonate (13a) (Typical Procedure)——The title compound (**13a**) was prepared from **12a** (2.0 g) and benzenesulfonic acid (3.5 g) in an ethereal solution (80 ml) in the same manner as described for **4a** (method A). Yield, 2.2 g (64%). Compounds **13b**—**f** were similarly prepared from the diazoketones (**12a**, **b**) and the corresponding arenesulfonic acids. Other data are listed in Tables VI and VII.

Enzyme-Inhibitory Activities——The inhibitory activities toward esterase and chymotrypsin were determined by the methods described in the previous paper.[5]

Pharmacology——Male Wistar rats weighing 200—220 g (7 weeks old) were used for the experiment. They were allocated to experimental groups of five animals. A test compound (0.3 mmol) was dissolved in olive oil (5 ml) and orally administered to the rats at the dose of 0.3 mmol/kg through a stomach tube. Blood samples were taken from the orbital vein under ether anesthesia at 2 h after administration. The samples were centrifuged at 3000 rpm at 5 °C to obtain the plasma. The triglyceride level in plasma was measured by using the Determiner TG-S Kyowa[16] (available from Kyowa Medex Co., Ltd., Japan). The control groups received only olive oil in the same manner, and the normal groups received no treatment. The plasma triglyceride levels of the control and normal groups were measured in the same manner. The percent reduction of the plasma triglyceride was calculated as follows:

$$\text{reduction } (\%) = \frac{A - C}{A - B} \times 100$$

A: plasma triglyceride level of the control group

B: that of the normal group

C: that of the group treated with the test compound

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Antiallergic Substance from *Asarum sagittarioides* and Synthesis of Some Analogues

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The MeOH extract of *Asarum sagittarioides* (Aristolochiaceae) showed antihistaminic activity. *N*-Isobutyl-3,4-methylenedioxybenzamide (**1**) was isolated as the active principle, and this compound was proved to be an antiallergic substance by pharmacological studies. Furthermore, in order to investigate the structure–activity relationships, some analogues of **1** were synthesized, and compounds **4** and **5** were found to show more potent activity than **1**.

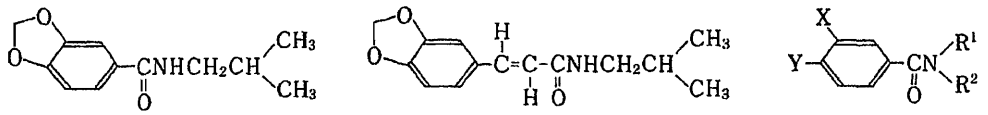
Keywords——benzamide; *Asarum sagittarioides*; Aristolochiaceae; antihistamine; antiallergic activity

In our project aimed at finding new biologically active compounds, extracts prepared from oriental drugs which have not previously been investigated chemically or pharmacologically were subjected to pharmacological screening tests. Among them, the extract of the Chinese crude drug "Shan ci gu (山慈姑)," showed strong antihistaminic activity.

Although "Shan ci gu," dry bulb of *Pleione* sp. (Orchidaceae), is said to be used as a mitigator of sore throat in China,[1] to our knowledge, no investigation of the active principles has been reported until now. Besides Orchidaceae plants, *Asarum sagittarioides* (Aristolochiaceae) and *Tinospora capillipis* (Menispermaceae) are also used for the same purpose in China.[2] The material for this study, which was purchased in Hong Kong, was considered to belong to *Asarum* sp. from the morphological characters.[3] It is likely that the material originated from *Asarum sagittarioides* in view of the region[2] where the crude drug was purchased.

The MeOH extract of the dried material relaxed a tracheal strip preparation contracted by histamine solution. The extract was partitioned between a mixture of EtOAc and H_2O. All activity was transferred to the EtOAc fraction, which exhibited only two components and some pigments on thin layer chromatography (TLC) examination. The fraction was subjected to silica gel column chromatography and high-performance liquid chromatography (HPLC) to afford AS-1 and AS-2.

AS-1 (**1**), mp 82.0—82.5 °C, $C_{12}H_{15}NO_3$, was the major component whose proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed the signals of an isobutyl group at δ 0.96 (6H, d, J=6.6 Hz), 1.88 (1H, m), and 3.24 (2H, dd, J=5.3, 5.9 Hz), a methylenedioxy group at δ 6.00 (2H, s), an amide proton at 6.27 (1H, exchangeable with D_2O), and aromatic protons at 6.80 (1H, d, J=8 Hz), 7.27 (1H, d, J=0.6 Hz), and 7.28 (1H, dd, J=0.6, 8 Hz). The infrared (IR) spectrum of **1** showed the absorption of an amide at 1635 cm^{-1} and the mass spectrum (MS) gave the base peak at m/z 149 suggesting the presence of piperonyl moiety. From these data, the structure of AS-1 was concluded to be *N*-isobutyl-3,4-methylenedioxybenzamide (**1**) and this was confirmed by the synthesis of the amide from piperonylic



1 2 3–17

Chart 1

TABLE I. Biological Properties of Benzamides (1—17)

Compd. No.	Antihistaminic effect[a]	Inhibitory effects	
		Rat PCA[b]	Schultz–Dale tests[c]
1	+	+	46 (%)
2	±	nt	nt
3	+	+	29
4	++	+	nt
5	++	+	25
6	+	+	nt
7	±	+	nt
8	+	+	17
9	±	+	33
10	+	+	29
11	+	+	13
12	+	+	nt
13	±	+	75
14	±	±	nt
15	+	+	22
16	+	+	31
17	+	+	8
18	nt	++	nt
19	nt	++	nt
20	nt	++	nt

a) The concentration of the sample was 5×10^{-5} g/ml. + relaxation of contraction caused by 10^{-8} M histamine solution; ++, relaxation of contraction caused by 10^{-6} M histamine solution; ±, no clear effect; nt, not tested. *b*) Samples were administered i.p. 1 h before challenge. Dose: **1**—**17**, 250 mg/kg; **18**, 30 mg/kg; **19**, 10 mg/kg; **20**, 60 mg/kg. The antiallergic activities of the test compounds were evaluated from the leakage of dye assessed with the naked eye. ++, strong inhibition; +, inhibition; ±, no clear effect, nt, not tested. *c*) The concentration of the sample was 50 μg/ml. nt, not tested.

acid and isobutylamine in the usual manner.

AS-2 (**2**), mp 101—102 °C, $C_{14}H_{17}NO_3$, showed the signals of a conjugated *trans* double bond (δ 6.30 and 7.53, each d, $J=16$ Hz) together with isobutyl and methylenedioxy groups in its ^{1}H-NMR spectrum. The MS of **2** exhibited the base peak at *m/z* 175, indicating the presence of a 3,4-methylenedioxyphenyl moiety. These data suggested the structure of AS-2 to be *N*-isobutyl-3,4-methylenedioxycinnamide, and this was confirmed by synthesis (Chart 1).

AS-2 (**2**) was identical with fagaramide[4] isolated from *Fagara macrophylla* (Rutaceae). Because cinnamic acid derivatives such as *N*-(*trans*-3,4-dimethoxycinnamoyl)anthranilic acid (tranilast)[5] (**18**) are known as antiallergic agents, **2** is not a new type of agent. In our experiments, **1** relaxed the tracheal plain muscle of guinea pig contracted with 5×10^{-7} and 5×10^{-8} M histamine solutions, while **2** exhibited only a slight relaxation of the muscle contraction caused by 5×10^{-8} M histamine solution.

Since histamine is an important chemical mediator in immediate-type allergy,[6] the effect of **1** on type I allergy[6] was investigated. The antiallergic activity on type I allergy was

TABLE II. Physicochemical Data for Derivatives of AS-1

Compd. No.	X	Y	R^1	R^2	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
								C	H	N
3	OH	OH	iso-C_4H_9	H	41.4	155.5—156.0	$C_{11}H_{15}NO_3$	63.14	7.23	6.69
								(63.01	7.15	6.45)
4[a]	OCH_3	OCH_3	iso-C_4H_9	H	52.0	120.0—121.0	$C_{13}H_{19}NO_3$	65.80	8.07	5.90
								(65.59	7.97	5.69)
5	OCH_3	OH	iso-C_4H_9	H	45.2	114.0—115.0	$C_{12}H_{17}NO_3$	64.55	7.68	6.27
								(64.41	7.52	6.14)
6	OC_2H_5	OC_2H_5	iso-C_4H_9	H	83.4	133.5—134.0	$C_{15}H_{23}NO_3$	67.89	8.74	5.28
								(67.70	8.58	5.08)
7	OC_2H_5	OC_2H_5	n-C_3H_7	H	88.2	127.5—128.0	$C_{14}H_{21}NO_3$	66.90	8.42	5.57
								(66.75	8.31	5.33)
8[a]	$-O-CH_2-O-$		n-C_3H_7	H	61.9	111.0—112.0	$C_{11}H_{13}NO_3$	63.75	6.32	6.76
								(63.70	6.24	6.59)
9[a]	$-O-CH_2-O-$		iso-C_3H_7	H	69.0	89.0—90.0	$C_{11}H_{13}NO_3$	63.75	6.32	6.76
								(63.66	6.30	6.80)
10[a]	$-O-CH_2-O-$		n-C_4H_9	H	68.7	70.0—71.0	$C_{12}H_{15}NO_3$	65.14	6.83	6.33
								(65.23	6.75	6.15)
11[a]	$-O-CH_2-O-$		sec-C_4H_9	H	80.6	81.5—82.5	$C_{12}H_{15}NO_3$	65.14	6.83	6.33
								(65.03	6.88	6.45)
12	$-O-CH_2-O-$		$-(CH_2)_2OH$	H	33.6	104.0—105.0	$C_{10}H_{11}NO_4$	57.41	5.30	6.70
								(57.24	5.32	6.55)
13	$-O-CH_2-O-$		$-(CH_2)_3OH$	H	17.0	119.0—120.0	$C_{11}H_{13}NO_4$	59.18	5.87	6.28
								(59.27	5.63	6.11)
14	$-O-CH_2-O-$		$-CH_2CH(OH)CH_3$	H	15.7	138.0—139.0	$C_{11}H_{13}NO_4$	59.18	5.87	6.28
								(59.35	5.65	6.09)
15	$-O-CH_2-O-$		Piperidyl		85.5	Oily	$C_{13}H_{15}NO_3$	66.93	6.48	6.01
								(66.87	6.37	6.03)
16	$-O-CH_2-O-$		Piperazinyl		51.4	280.0—281.0	$C_{12}H_{14}N_2O_3$	61.52	6.02	11.96
								(61.35	5.83	12.09)
17	$-O-CH_2-O-$		Morpholinyl		89.7	Oily	$C_{12}H_{13}NO_4$	61.27	5.57	5.96
								(61.41	5.69	5.80)

a) Known compound.[7]

evaluated from the inhibitory activity on the Schultz–Dale (*in vitro*) and passive cutaneous anaphylaxis (PCA) (*in vivo*) reactions.

In the Schultz–Dale test, **1** (5×10^{-5} g/ml) inhibited the contraction of the ileum by antigen. AS-1 (**1**) also inhibited the leakage of pigments in the PCA test (Table I). Thus, **1** is considered to be the active principle in *Asarum sagittarioides*.

Although many kinds of piperonyl amide derivatives including AS-1 have been synthesized because of their extensive insecticidal effects,[7] there is no report concerning antiallergic activity. However, commercially available antiallergic agents such as tranilast (**18**), ketotifen fumarate (**19**),[8] and tiaramide HCl (**20**),[9] inhibited the PCA more strongly than **1** as shown in Table I.

We attempted to produce a more effective compound based on the structure of **1**. In order to investigate the influence of *N*-substituents on the antiallergic activity, a variety of *N*-alkyl analogues of **1** (compounds **8** to **17**) were synthesized. The physical and spectral data of these compounds are given in Tables II and III, respectively.

Examination of the antihistaminic effect of these compounds suggested that: (1) an *N*-alkyl chain containing at least three carbon atoms is indispensable for the appearance of

TABLE III. Spectral Data for Derivatives of AS-1

Compd. No.	MS (*m/z*)	IR (cm^{-1})	^{1}H-NMR δ ($CDCl_3$)
3	209 (M^+)	3510, 1620	[a] 8.39 (1H, brs), 7.66 (1H, brs), 7.48 (1H, d, J=2.3 Hz), 7.32 (1H, dd, J=2.3, 8.4 Hz), 6.85 (1H, d, J=8.3 Hz), 3.19 (2H, t, J=6.9 Hz), 1.91 (1H, m), 0.92 (6H, d, J=6.6 Hz)
4	237 (M^+)	3300, 1630	7.43 (1H, d, J=2.0 Hz), 7.25 (1H, dd, J=2.3, 8.3 Hz), 6.86 (1H, d, J=8.3 Hz), 6.15 (1H, brs), 3.93 (3H, s), 3.92 (3H, s), 3.28 (2H, t, J=6.2 Hz), 1.90 (1H, m), 0.98 (6H, d, J=6.6 Hz)
5	223 (M^+)	3300, 1635	7.46 (1H, d, J=2.0 Hz), 7.18 (1H, dd, J=2.0, 8.3 Hz), 6.91 (1H, d, J=8.3 Hz), 6.15 (1H, brs), 3.93 (3H, s), 3.27 (1H, t, J=6.3 Hz), 1.89 (1H, m), 0.97 (6H, d, J=6.9 Hz)
6	251 (M^+)	3280, 1625	7.41 (1H, d, J=2.0 Hz), 7.22 (1H, dd, J=2.3, 8.9 Hz), 6.86 (1H, d, J=8.6 Hz), 6.08 (1H, br s), 4.14 (4H, q, J=7.1 Hz), 3.27 (2H, t, J=7.0 Hz), 1.89 (1H, m), 1.47 (6H, t, J=7.1 Hz), 0.98 (6H, d, J=7.0 Hz)
7	237 (M^+)	3280, 1630	7.41 (1H, d, J=2.3 Hz), 7.22 (1H, dd, J=2.3, 8.5 Hz), 6.85 (1H, d, J=8.2 Hz), 6.07 (1H, brs), 4.14 (4H, q, J=7.2 Hz), 3.40 (2H, q, J=7.0 Hz), 1.65 (1H, m), 1.47 (6H, t, J=7.1 Hz), 0.98 (3H, d, J=7.3 Hz)
8	207 (M^+)	3290, 1630	7.30 (1H, d, J=1.6 Hz), 7.26 (1H, d, J=1.6 Hz), 6.81 (1H, dd, J=1.0, 7.3 Hz), 6.12 (1H, brs), 6.01 (2H, s), 3.38 (2H, m), 1.62 (2H, m), 0.98 (3H, t, J=7.2 Hz)
9	207 (M^+)	3300, 1630	7.26 (2H, m), 6.81 (1H, d, J=8.3 Hz), 6.01 (2H, s), 5.83 (1H, brs), 4.24 (1H, m), 1.24 (6H, d, J=6.6 Hz)
10	221 (M^+)	3340, 1640	7.28 (2H, m), 6.81 (1H, dd, J=8.6 Hz), 6.10 (1H, brs), 6.01 (2H, s), 3.41 (2H, q, J=6.9 Hz), 1.58 (2H, m), 1.39 (2H, m), 0.96 (3H, t, J=7.0 Hz)
11	221 (M^+)	3260, 1635	7.28 (2H, m), 6.81 (1H, d, J=8.9 Hz), 6.01 (2H, s), 5.83 (1H, brs), 4.09 (1H, m), 1.56 (2H, quintet, J=7.0 Hz), 1.21 (3H, d, J=6.6 Hz), 0.94 (3H, t, J=7.2 Hz)
12	209 (M^+)	3450, 1650	[b] 7.85 (2H, m), 6.92 (1H, d, J=8.3 Hz), 5.98 (2H, s), 4.13 (2H, t, J=6.0 Hz), 3.93 (2H, q, J=6.0 Hz)
13	223 (M^+)	3280, 1630	7.30 (1H, dd, J=1.7, 7.9 Hz), 7.26 (1H, d, J=2.0 Hz), 6.81 (1H, d, J=8.2 Hz), 6.64 (1H, brs), 6.02 (2H, s), 3.71 (2H, q, J=5.5 Hz), 3.60 (2H, q, J=6.0 Hz), 1.78 (2H, quintet, J=6.0 Hz)
14	223 (M^+)	3310, 3220, 1635	8.99 (1H, brs), 7.86 (2H, m), 6.91 (1H, d, J=8.8 Hz), 5.98 (2H, s), 4.41 (1H, m), 3.90 (1H, m), 3.74 (1H, m), 1.39 (3H, d, J=6.4 Hz)
15	233 (M)	1620	6.91 (1H, dd, J=1.3, 7.9 Hz), 6.89 (1H, d, J=1.7 Hz), 6.81 (1H, d, J=7.6 Hz), 5.99 (2H, s), 3.51 (4H, br)
16	234 (M^+)	3440, 1620	6.94 (1H, dd, J=1.3, 7.9 Hz), 6.92 (1H, d, J=2.0 Hz), 6.83 (1H, d, J=7.9 Hz), 6.01 (2H, s), 3.64 (4H, br)
17	235 (M^+)	1625	6.93 (1H, dd, J=1.6, 7.7 Hz), 6.91 (1H, d, J=1.3 Hz), 6.83 (1H, d, J=7.6 Hz), 6.00 (2H, s), 3.65 (8H, m)

a) Acetone-d_6 was used as a solvent. *b*) Pyridine-d_5 was used as a solvent.

activity; (2) a hydroxy group on the *N*-alkyl chain decreases the activity. An *N*-substituent which increased the activity of **1** was not discovered in this experiment.

Next, we modified the benzene ring of **1**. Four kinds of carboxylic acids were transformed to the corresponding isobutyl amide derivatives (compounds **3** to **6**), and they were subjected to bioassay. Among them, the amides of veratric and vanillic acids (**4** and **5**) relaxed the trachea plain muscle contracted by 5×10^{-6} M histamine solution, and were more potent than **1**.

It seems likely that attempts to increase the antiallergic activity of **1** by modification of the benzamide should center on the benzene ring.

In the PCA tests, inhibition of the pigment leakage by ketotifen fumarate (**19**) was much

stronger than that by **4** or **5**. Further studies are in progress.

Experimental

Melting points were determined with a Kofler-type apparatus and were uncorrected. IR spectra were recorded in KBr discs on a Hitachi 260-10 infrared spectrometer and ultraviolet (UV) spectra with Hitachi 340 and 356 spectrometers. ^{1}H-NMR spectra were taken on a a JEOL FX-270 spectrometer with tetramethylsilane as an internal standard, and the following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br s=broad singlet, dd=double doublet. MS were recorded on JEOL D-300 and Hitachi M-60 spectrometers. HPLC was run on a Hitachi 635A instrument with a UV detector (conditions: LiChrosorb RP-18, 8 mm i.d. × 250 mm; flow rate, 2 ml/min).

Isolation of AS-1 (1) and AS-2 (2)——The pulverized crude drug (250 g) was extracted with MeOH at room temperature. The methanolic solution was concentrated *in vacuo* and the residue was partitioned between a mixture of EtOAc–H_2O. Evaporation of EtOAc solution to dryness yielded 3.55 g of greenish brown oil. The EtOAc extract showing antihistamine activity was chromatographed on silica gel with a $CHCl_3$–MeOH gradient system. The fractions, fr. 1 and fr. 2, eluted with 2% MeOH–$CHCl_3$ contained the main components of the EtOAc-soluble fraction. Recrystallization of fr. 1 gave **2** (28.5 mg), colorless needles. Fraction 2 was rechromatographed on silica gel with $CHCl_3$–*n*-hexane (1 : 3) as the eluent and further purified by HPLC to afford **1** (40 mg), colorless needles.

AS-1 (1)——Colorless needles from *n*-hexane–$CHCl_3$, mp 82.0—82.5 °C. MS *m/z*: 221 (M^+), 149 (piperonyl moiety, base peak). IR ν_{max}^{KBr} cm^{-1}: 3300, 1635, 1535, 1480, 1355, 1315, 1260, 1040. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (6H, d, J=6.6 Hz), 1.88 (1H, m), 3.24 (2H, dd, J=5.3, 5.7 Hz), 6.00 (2H, s), 6.27 (1H, br s), 6.80 (1H, d, J=8 Hz), 7.27 (1H, d, J=0.6 Hz), 7.28 (1H, dd, J=0.6, 8 Hz). UV λ_{max}^{MeOH} nm (log ε): 255 (3.87), 289 (3.80). *Anal.* Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.01; H, 6.97; N, 6.12.

AS-2 (2)——Colorless needles from *n*-hexane–$CHCl_3$, mp 101—102 °C. MS *m/z*: 247 (M^+), 175 (base peak). IR ν_{max}^{KBr} cm^{-1}: 3280, 3075, 2950, 1645, 1605, 1550. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (6H, d, J=6.6 Hz), 1.84 (1H, m), 3.21 (2H, t, J=6.6 Hz), 6.30 (1H, d, J=15.5 Hz), 6.76 (1H, d, J=8 Hz), 6.95 (1H, dd, J=1.6, 8 Hz), 6.98 (1H, d, J=1.6 Hz), 7.53 (1H, d, J=15.5 Hz). UV λ_{max}^{MeOH} nm (log ε): 228 (4.21), 284 (4.22), 318 (4.29). *Anal.* Calcd for $C_{14}H_{17}NO_3$: C, 67.99; H, 6.93; N, 5.66. Found: C, 68.05; H, 6.70; N, 5.42.

Synthesis of 3,4-Methylenedioxycinnamic Acid——A mixture of piperonal (7.5 g), acetic anhydride (7.1 ml), and potassium acetate (2.94 g) was refluxed for 6 h. After cooling, the reaction mixture was resolved in 1 N NaOH and extracted with Et_2O. The lower layer was acidified with 1 N HCl and the precipitate was recrystallized from hot EtOH to afford light yellow needles (yield, 50%). The crystals, mp 244—245 °C, showed the signals of a *trans*-conjugated double bond at δ 6.88 (1H, d, J=16 Hz) and 8.00 (1H, d, J=16 Hz) in the ^{1}H-NMR spectrum.

General Procedure of Amide Synthesis——A solution of 0.5 g of the acid, 2 ml of thionyl chloride, and 10 ml of 1,2-dichloroethane was heated under reflux for 1 h. After evaporation of the solvent, the residue was dissolved in 2 ml of dry pyridine. The solution was added portionwise to 5 ml of the amine with stirring and ice-cooling. The resulting solution was stirred for 2 h under ice-cooling, then poured into H_2O and extracted with $CHCl_3$. The organic layer was washed with 1 N HCl, brine, and H_2O successively and dried with anhydrous Na_2SO_4. Evaporation of the solvent yielded the product, which was recrystallized from a suitable solvent.

Antihistaminic Effect——The procedure is a modification of that described by Macleod.[10] Male Hartley guinea pigs weighing approximately 350 g were killed and tracheal strip chain preparations were made. The preparations were placed in a tissue-organ bath containing Tyrode solution and aerated with a gas mixture (O_2 : CO_2 = 95 : 5). The relaxing effects of the test compounds were measured from a kymographic recording of contraction of the tracheal preparation induced by histamine solution (5×10^{-8} and 5×10^{-6} M). The results were expressed as follows: − (contraction); ± (no clear effect); + (weak relaxation); + + (strong relaxation).

Passive Cutaneous Anaphylaxis (PCA)——PCA tests were carried out, according to the method of Ovary *et al.*[11] Female Hartley guinea pigs weighing 350—400 g were passively sensitized by injecting i.c. on the back 28 μg/0.1 ml of antichicken egg albumin. After 3 h, they were challenged by injecting i.p. 0.5 ml of 1% Evans blue saline solution containing 50 μg of egg albumin. Test compounds (at the doses shown in Table I) were suspended in 5% EtOH containing 5% Macrogol 400, and administered to sensitized guinea pig 1 h before challenge. The antiallergic activities of the test compounds were evaluated from the degree of leakage of pigments assessed with the naked eye: + + (strong inhibition); + (inhibition); ± (no clear effect); − (inactive). This evaluation method is not as satisfactory as the method[12] by which the extracted dye is determined as an absolute amount.

Schultz–Dale Test——Male Hartley guinea pigs weighing *ca.* 300 g were sensitized by injecting i.p. 100 μg/kg/ml of egg albumin in accordance with the method of Fujimura *et al.*[13] Twelve days later, the animals were guillotined and the ileum were isolated. The preparation was contracted by exposure to 5×10^{-8} M histamine (A), 100 μg/ml of antigen (B) and the solution containing the test compound and 100 μg/ml of antigen (C). The percent inhibition was estimated by means of the following formula.

$$\text{Inhibition } (\%) = (1 - y/x) \times 100$$

$$x=\frac{\text{strength of contraction by B}}{\text{strength of contraction by A}}, \qquad y=\frac{\text{strength of contraction by C}}{\text{strength of contraction by A}}$$

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Constituents of Pollen. XIV.[1] Constituents of *Cedrus deodara* LOUD. (3)

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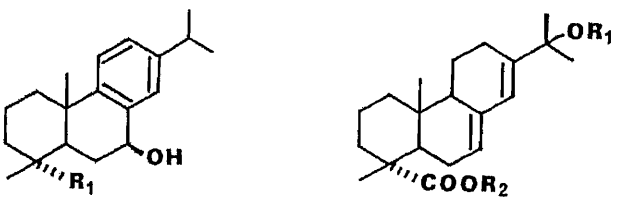
Three new compounds have been isolated from the pollen grains of *Cedrus deodara* LOUD. and these compounds were determined to be 7β,18-dihydroxydehydroabietanol (III), 15-methoxyabietic acid (IV) and 9-caffeoyloxyhexadecanol (V) by chemical and spectroscopic methods. 7β-Hydroxydehydroabietic acid (I) and 15-hydroxyabietic acid (II) were also isolated.

Keywords——*Cedrus deodara*; Pinaceae; pollen grains; 7β,18-dihydroxydehydroabietanol; 15-methoxyabietic acid; 9-caffeoyloxyhexadecanol

In the previous paper,[1] we reported the isolation of several diterpenoids, a caffeoyl ester, naringenin and β-sitosteryl β-D-glucoside, from the pollen grains of *Cedrus deodara* LOUD. (Himarayasugi in Japanese) (Pinaceae). Further investigation of the chemical constituents of these pollen grains resulted in the isolation of two new diterpenoids and a new caffeoyl ester, along with two known compounds. This paper deals with the isolation and structural elucidation of these compounds.

The ether extract of the pollen grains of *Cedrus deodara* was chromatographed on silica gel to yield compounds I—V. Compounds I and II were identified as 7β-hydroxydehydroabietic acid and 15-hydroxyabietic acid, respectively, by comparisons of their physical and spectral data with those of authentic samples.[2,3]

Compound III was obtained as a white powder. The high-resolution mass spectral examination of III gave the formula $C_{20}H_{30}O_2$. The ultraviolet (UV) spectrum exhibited absorption maxima at 216, 264 and 276 nm, and the infrared (IR) spectrum showed absorption due to a hydroxyl group at 3450 cm^{-1}. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed methyl signals at δ 0.89 (3H, s), 1.24 (3H×2, d, J=7 Hz) and 1.29 (3H, s), and hydroxymethyl signals at δ 3.22 (1H, d, J=11 Hz) and 3.49 (1H, d, J=11 Hz). Aromatic proton signals appeared as an ABX system at δ 7.10 (1H, dd, J=8, 2 Hz), 7.17 (1H, d, J=8 Hz) and 7.37 (1H, d, J=2 Hz). Furthermore, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of III showed rather similar chemical shifts to those



I: R_1=COOH
III: R_1=CH_2OH

II: R_1=R_2=H
IIa: R_1=H, R_2=CH_3
IV: R_1=CH_3, R_2=H

Fig. 1

Table I. ^{13}C-NMR Chemical Shifts of I—IV

Carbon No.	I	II	III	IV
1	38.0	38.1	38.4	38.4
2	18.7	18.6	18.5	18.5
3	33.7	37.7	34.8	37.6
4	36.4	46.1	38.0	46.3
5	47.3	50.5	42.5	50.1
6	29.7	26.9	30.0	26.0
7	70.7	121.9	70.8	123.9
8	137.7	134.4	137.6	132.2
9	146.9	44.7	147.2	44.4
10	37.6	34.3	37.5	34.2
11	125.2	18.1	125.0	18.6
12	124.2	38.5	124.4	38.9
13	146.1	143.8	146.4	141.4
14	125.5	122.3	125.8	126.2
15	33.4	72.5	33.7	72.9
16	24.0	28.6	24.1	29.5
17	24.0	28.5	23.9	29.4
18	181.4	183.0	71.8	182.8
19	16.8	17.2	17.4	16.4
20	25.6	14.6	25.8	14.2
$-OCH_3$				51.9

Run at 100 MHz in $CDCl_3$ solution.

of 7α,18-dihydroxydehydroabietanol,[1] which has already been isolated from these pollen grains. These data suggested that III has a dehydroabietane skeleton. The β-configuration of the hydroxy group at C-7 of III was determined from the ^{1}H-NMR spectrum, in which a 1H triplet at δ 4.85 (J=8 Hz) due to a hydroxymethine proton was observed.[4] This conclusion was confirmed by the difference spectrum of nuclear Overhauser effect (NOE), in which increments of the signal intensity of H-6 (δ 2.20) and H-14 (δ 7.37) were observed when H-7 was irradiated. From these spectral correlations, III was determined to be 7β,18-dihydroxydehydroabietanol.

Compound IV was obtained as a white powder. The high-resolution mass spectral examination of IV gave the formula $C_{21}H_{32}O_3$. The UV spectrum exhibited absorption maxima at 234, 241 and 248 nm and the IR spectrum showed absorption due to a carbonyl group at 1640 cm^{-1}. Comparison of the ^{1}H- and ^{13}C-NMR spectra of IV with those of II indicated that both compounds possess the same skeleton (Table I). The presence of a methoxy group in IV was indicated by the signal at δ 3.02 in the ^{1}H-NMR spectrum. On the other hand, II was methylated with diazomethane to give IIa, which was not identical with IV. The mass spectrum (MS) showed the molecular ion peak (M^+) at m/z 332 with fragment ion peaks of $M^+ - OCH_3$ at m/z 301 and a methoxyl isopropyl group at m/z 73. Consequently, IV was determined to be 15-methoxyabietic acid.

Compound V was obtained as a yellow oil, which was positive to the ferric chloride reaction. The high-resolution mass spectral examination of III gave the formula $C_{25}H_{40}O_5$. The UV spectrum showed absorption maxima at 220, 236, 246, 300 and 332 nm and the IR spectrum showed absorptions due to a hydroxyl group at 3400 cm^{-1} and an ester at 1710 and 1270 cm^{-1}. The ^{1}H-NMR spectrum showed methylene protons at δ 1.25 (br s), primary alcoholic protons at δ 3.64 (2H, t, J=7 Hz), a methine proton at δ 4.98 (1H, t, J=7 Hz), protons attached to a 1,2,4-trisubstituted benzene ring at δ 6.85 (1H, d, J=7 Hz), 6.95 (1H, dd, J=7, 2 Hz) and 7.08 (1H, d, J=2 Hz) and *trans* olefinic protons at δ 6.22 (1H, d, J=

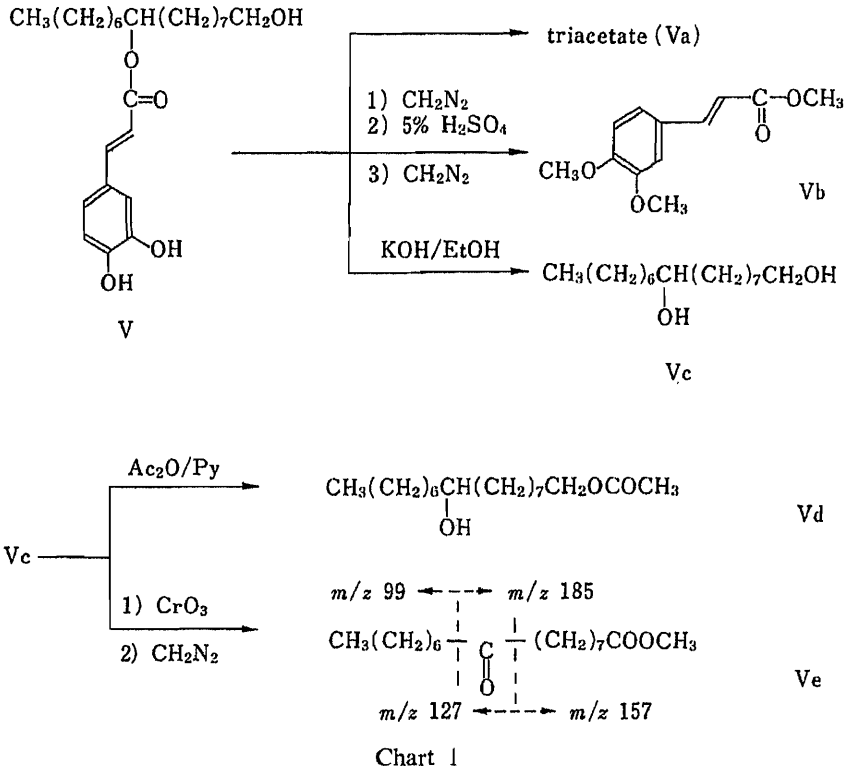


Chart 1

14 Hz) and 7.54 (1H, d, J=14 Hz). The above spectroscopic data were similar to those of hexadecane-1,16-diol 7-caffeoyl ester,[1] which has already been isolated from these pollen grains. When treated with acetic anhydride in pyridine, V gave a triacetate (Va) as a colorless oil. Thus, V was deduced to be an ester of caffeic acid with a secondary hydroxyl group of an aliphatic diol. Compound V was methylated with diazomethane and the product was hydrolyzed with 5% H_2SO_4. The hydrolysate was methylated with diazomethane to afford Vb. Compound Vb was identified as 3,4-dimethoxycaffeic acid methyl ester by comparison with an authentic sample on thin layer chromatography (TLC) and gas liquid chromatography (GLC). On the other hand, alkaline hydrolysis of V gave Vc as a white powder. Compound Vc was acetylated by the conventional method to give a monoacetate (Vd) as a colorless oil. Compound Vc was oxidized with chromium trioxide and then methylated with diazomethane to give Ve as a colorless oil. The MS of Ve showed M^+ at m/z 284 with fragment ion peaks at m/z 185, 157, 127 and 99 as shown in Chart 1. Therefore, Vc was determined to be hexadecane-1,9-diol. These results indicated that caffeic acid might be linked by an ester bond to C-9 of Vc. Thus, V was determined to be 9-caffeoyloxyhexadecanol.

In conclusion, five compounds (I—V) were isolated for the first time from the pollen grains of *Cedrus deodara*.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The UV and IR spectra were recorded with Hitachi 340 and Hitachi 260-30 spectrophotometers, respectively. The ^1H- and ^{13}C-NMR spectra were recorded with JEOL GX-400 (^1H, 400 MHz; ^{13}C, 100 MHz) spectrometers. Chemical shifts are given on the δ scale (ppm) with tetramethylsilane as an internal standard, and coupling constants are given in Hz. The following abbreviations are used: s=singlet, br s=broad singlet, d=doublet, dd=double doublet, t=triplet, q=quartet, hep=heptet and m=multiplet. The MS and optical rotations were measured with a JEOL JMS-01-SG-2 mass spectrometer and a JASCO DIP-4 digital polarimeter, respectively. GLC was carried out on a Hitachi 063 gas liquid chromatograph using a stainless steel column (3 mm×1 m) packed with 2% SE-30 and 10% SE-30 on

Chromosorb-W (60—80 mesh) with N_2 carrier gas at a flow rate of 30 ml/min. Column chromatography was performed on silica gel [Fuji-Davison BW-820 MH]. TLC was carried out on precoated Silica gel 60 F-254 plates (Merck) and the spots were detected by using 5% $FeCl_3$ or 10% H_2SO_4.

Extraction and Isolation——Pollen grains (2174 g) of *Cedrus deodara*, collected in November, 1984, at Toho University, were extracted with ether in a Soxhlet apparatus for 30 h. The extract (309 g) was applied to a column of silica gel (1.5 kg) and eluted successively with hexane, hexane–benzene (1 : 1), benzene, benzene–$CHCl_3$ (1 : 1), $CHCl_3$, $CHCl_3$–MeOH (1 : 1) and MeOH. The benzene–$CHCl_3$ (1 : 1) fraction was repeatedly chromatographed on silica gel to afford III (12.7 mg). The $CHCl_3$ fraction was repeatedly chromatographed on silica gel to afford I (10.7 mg), II (20.1 mg) and IV (8.6 mg). The $CHCl_3$–MeOH (1 : 1) fraction was repeatedly chromatographed on silica gel to afford V (75.2 mg).

7β-Hydroxydehydroabietic Acid (I)——A white powder. $[\alpha]_D^{20}$ +25.6° (c=0.5, EtOH). High-resolution MS *m/z*: Calcd for $C_{20}H_{28}O_3$: 316.2038. Found: 316.2073. UV λ_{max}^{EtOH} nm (log ε): 226 (3.37), 252 (2.90), 276 (2.65). IR ν_{max}^{KBr} cm^{-1}: 3420, 1690, 1550. MS *m/z*: 316 (16%, M$^+$), 298 (84), 283 (33), 237 (100), 197 (94), 195 (88), 162 (49), 155 (35), 55 (24), 43 (48). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.19 (3H, s), 1.20 (3H×2, br s), 1.25 (3H, s), 2.82 (1H, br s), 4.82 (1H, br s), 7.05 (1H, dd, J=8, 2 Hz), 7.08 (1H, d, J=8 Hz), 7.33 (1H, d, J=2 Hz). ^{13}C-NMR: Table I.

15-Hydroxyabietic Acid (II)——A white powder. $[\alpha]_D^{20}$ −16.7° (c=1.5, EtOH). *Anal.* Calcd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.40. Found: C, 75.64; H, 9.71. UV λ_{max}^{EtOH} nm (log ε): 234 (3.37), 241 (3.47), 248 (3.25). IR ν_{max}^{KBr} cm^{-1}: 3450, 1690, 1470, 1420. MS *m/z*: 318 (39%, M$^+$), 303 (63), 300 (100), 285 (15), 275 (19), 134 (39), 121 (43), 59 (16), 43 (59). ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.82 (3H, s), 1.25 (3H, s), 1.32 (3H, s), 1.34 (3H, s), 5.47 (1H, br s), 6.06 (1H, d, J=2 Hz). ^{13}C-NMR: Table I.

Methylation of II——II was dissolved in ether and methylated with diazomethane at room temperature for 1 h to give the monomethylester (IIa), methyl 15-hydroxyabietate, as a colorless oil. MS *m/z*: 332 (M$^+$). IR $\nu_{max}^{liq.}$ cm^{-1}: 3450, 1720, 1460, 1250. ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.82 (3H, s), 1.22 (3H, s), 1.25 (3H×2, s), 3.68 (3H, s), 5.42 (1H, m), 5.98 (1H, m).

7β,18-Dihydroxydehydroabietanol (III)——A white powder. $[\alpha]_D^{20}$ +38° (c=0.2, EtOH). High-resolution MS *m/z*: Calcd for $C_{20}H_{30}O_2$: 302.2246. Found: 302.2264. UV λ_{max}^{EtOH} nm (log ε): 216 (4.23), 264 (3.53), 276 (3.08). IR ν_{max}^{KBr} cm^{-1}: 3450, 1475, 1382, 1050, 730. MS *m/z*: 302 (48%, M$^+$), 254 (5), 251 (76), 239 (35), 209 (47), 197 (17), 162 (100), 55 (14), 43 (34). ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.89 (3H, s), 1.24 (3H×2, d, J=7 Hz), 1.29 (3H, s), 1.69 (1H, m), 1.82 (1H, m), 2.20 (1H, dd, J=12, 8 Hz), 2.87 (1H, hep), 3.22 (1H, d, J=11 Hz), 3.49 (1H, d, J=11 Hz), 4.85 (1H, t, J=8 Hz), 7.10 (1H, dd, J=8, 2 Hz), 7.17 (1H, d, J=8 Hz), 7.37 (1H, d, J=2 Hz). ^{13}C-NMR: Table I.

15-Methoxyabietic Acid (IV)——A white powder. $[\alpha]_D^{20}$ −25.0° (c=0.2, EtOH). High-resolution MS *m/z*: Calcd for $C_{21}H_{32}O_3$: 332.2351. Found: 332.2386. UV λ_{max}^{EtOH} nm (log ε): 234 (3.77), 241 (3.78), 248 (3.62). IR ν_{max}^{KBr} cm^{-1}: 1640, 1460, 1380. MS *m/z*: 332 (33%, M$^+$), 318 (34), 317 (100), 301 (33), 300 (50), 285 (35), 73 (19), 59 (19), 55 (42), 43 (51), 31 (13). ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.82 (3H, s), 1.25 (3H, s), 1.29 (3H×2, s), 3.02 (3H, s), 5.45 (1H, br s), 5.93 (1H, br s). ^{13}C-NMR: Table I.

9-Caffeoyloxyhexadecanol (V)——A yellow oil, $[\alpha]_D^{20}$ −2.1° (c=2.5, MeOH). High-resolution MS *m/z*: Calcd for $C_{25}H_{40}O_5$: 420.2876. Found: 420.2886. UV λ_{max}^{EtOH} nm (log ε): 220 (4.09), 236 (3.89), 246 (3.91), 300sh (4.00), 332 (4.13). IR $\nu_{max}^{liq.}$ cm^{-1}: 3400, 2850, 1710, 1690, 1600, 1510, 1440, 1360, 1270. MS *m/z*: 420 (2%, M$^+$), 241 (20), 180 (100), 173 (9), 163 (28), 137 (10), 95 (26), 81 (19), 69 (27), 57 (26), 55 (29), 43 (32). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.25 (br s), 3.64 (2H, t, J=7 Hz), 4.98 (1H, t, J=7 Hz), 6.22 (1H, d, J=14 Hz), 6.85 (1H, d, J=7 Hz), 6.95 (1H, dd, J=7, 2 Hz), 7.08 (1H, d, J=2 Hz), 7.54 (1H, d, J=14 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 168.0 (C-9'), 147.0 (C-4'), 145.1 (C-3'), 144.5 (C-7'), 127.2 (C-1'), 122.2 (C-6'), 115.6 (C-5'), 115.4 (C-8'), 114.2 (C-2'), 74.7 (C-9), 62.9 (C-1), 21.0 (C-16).

Acetylation of V——V (18 mg) was acetylated with Ac_2O (2 ml) in pyridine (0.5 ml) at room temperature for 14 h to afford a triacetate (Va) as a colorless oil (15 mg). MS *m/z*: 546 (M$^+$), 341, 180 (base), 162. ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.25 (br s), 2.01 (3H, s, $-OCOCH_3$), 2.28 (3H×2, s, $-OCOCH_3$), 4.03 (2H, t, J=6 Hz), 4.98 (1H, t, J=6 Hz), 6.36 (1H, d, J=15 Hz), 7.19 (1H, dd, J=9, 2 Hz), 7.35 (1H, d, J=9 Hz), 7.37 (1H, d, J=2 Hz), 7.60 (1H, d, J=15 Hz).

Identification of 3,4-Dimethoxycaffeic Acid Methyl Ester (Vb)——V (10 mg) was dissolved in ether and methylated with diazomethane at room temperature overnight. Then the product was refluxed for 3 h in EtOH (2 ml) containing 10% H_2SO_4 (2 ml). The reaction mixture was extracted with ether. Then the ether extract was methylated with diazomethane to give 3,4-dimethoxycaffeic acid methyl ester (Vb), which was shown to be identical with an authentic sample by TLC and GLC.

Hydrolysis of V——V (20 mg) was refluxed for 3 h in EtOH (5 ml) containing KOH (0.5 g). Then the reaction mixture was acidified with dil. HCl and extracted with ether. The extract gave hexadecane-1,9-diol (Vc) as a white powder. MS *m/z*: 258 (M$^+$), 243, 227, 129 (base), 99. ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.28 (br s), 3.64 (2H, t, J=7 Hz).

Acetylation of Vc——Vc (6 mg) was acetylated with Ac_2O (2 ml) in pyridine (0.5 ml) at room temperature for 10 h to afford a monoacetate (Vd) as a colorless oil (5.8 mg). MS *m/z*: 300 (2%, M$^+$), 257 (7), 201 (9), 173 (40), 127 (9), 99 (13), 43 (100), 18 (5). ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.27 (br s), 1.62 (br s), 2.01 (3H, s, $-OCOCH_3$), 4.03 (2H, t, J=6 Hz), 4.85 (1H, t, J=6 Hz).

Oxidation of Vc——Vc (3 mg) was dissolved in pyridine (0.5 ml) and allowed to stand with CrO_3–pyridine (15 mg

in 1 ml) overnight at room temperature, then the mixture was poured into aq. MeOH. 9-Oxo-hexadecanoic acid was given as colorless oil. Then the product was methylated with diazomethane at room temperature for 3 h to give methyl 9-oxo-hexadecanoate (Ve) as a colorless oil (1.2 mg). MS *m/z*: 284 (3%, M^+), 185 (22), 157 (45), 149 (100), 127 (31), 99 (28).

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References and Notes

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Preparation of [1,2-^{3}H,4-^{14}C]16α-Hydroxyandrostenedione and Its Use for Radiometric Determination of Human Placental Aromatase Activity

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[1,2-^{3}H, 4-^{14}C]16α-Hydroxyandrostenedione (**4**) (^{3}H, 3.20 mCi/mmol; ^{3}H/^{14}C=222) was synthesized from commercially available [1,2-^{3}H, 4-^{14}C]dehydroepiandrosterone (**1**) through bromination at C-16α of the 17-ketone **1** and controlled alkaline hydrolysis of the 16α-bromoketone **3**, obtained from the brominated product **2** by 8 N CrO_3 oxidation followed by *p*-toluenesulfonic acid treatment, as key reactions. The tritium distribution of the labeled 16α-ketol **4** was determined by chemical and biochemical methods to be 47% at C-1α, 18% at C-2α, and 35% at the β-side of C-1 and C-2. When the labeled ketol **4** was incubated with human placental microsomes and reduced nicotinamide adenine dinucleotide phosphate, the rate of ^3H_2O release into the medium was dependent upon protein concentration and incubation time. Aromatase activity obtained by the radiometric assay was comparable to that determined by the high-performance liquid chromatographic method.

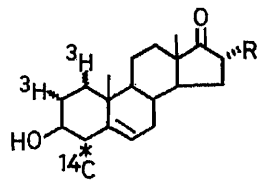
Keywords——[1,2-^{3}H, 4-^{14}C]16α-hydroxyandrostenedione; chemical synthesis; human placenta; aromatase; aromatization; estriol biosynthesis; tritiated water; radiometric assay

Aromatase is capable of catalyzing the synthesis of estrogens from androgens. There are certain structural variations in the androgen precursors for naturally occurring estrogen.[1] Two distinct aromatases (I and II) which have different substrate specificities and cytochrome P-450s have recently been separated in soluble form from human term placenta by Osawa's group.[2] The difference in the substrate specificity lies in the D-ring structure.

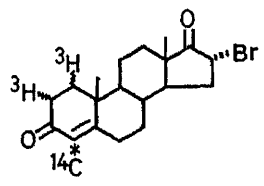
Aromatase II activities have usually been determined by radiometric methods based upon the stereospecific removal of tritium from [1β,2β-^{3}H]androstenedione[3] to ^3H_2O during aromatization.[4] This assay is highly sensitive, potentially accurate and speedy. On the other hand, we[5] have recently developed a sensitive high-performance liquid chromatographic (HPLC) method using 16α-hydroxyandrostenedione as a substrate for the determination of aromatase I activities. However, this chromatographic method is not as simple and speedy as the ^3H_2O release method. Thus it seems to be important to develop a convenient and rapid radiometric ^3H_2O assay method for the determination of aromatase I activities. We planned to synthesize [1,2-^{3}H, 4-^{14}C]16α-hydroxyandrostenedione (**4**) and to validate its use for the aromatase activity determination of human placental microsomes.

Results and Discussion

[1,2-^{3}H, 4-^{14}C]16α-Hydroxyandrostenedione (**4**) was chemically synthesized essentially according to our previous report.[6] [1,2-^{3}H]Dehydroepiandrosterone (DHEA, **1**) was mixed with [4-^{14}C]DHEA and diluted with non-labeled DHEA. The double labeled DHEA (^{3}H/



1 : R=H
2 : R=Br



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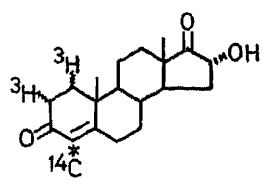

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Chart 1

TABLE I. Determination of Radiochemical Purity [1,2-^{3}H,4-^{14}C]Compound **4** by the Reverse Isotope Dilution Method[a]

No.	Crystallization From	Specific activity (dpm/mg) ^{3}H	^{14}C	^{3}H/^{14}C
1	Acetone	67550	302	224
2	Acetone	67400	305	221
3	MeOH–H_2O	67620	305	222

a) [1,2-^{3}H,4-^{14}C]Compound **4** (^{3}H, 1.36×10^6 dpm; ^{14}C, 6.14×10^3 dpm) was diluted with non-labeled compound **4** (20 mg) and repeatedly recrystallized.

TABLE II. Determination of ^{3}H- to-^{14}C Ratio of Estrogens Formed from [1,2-^{3}H,4-^{14}C]Compound **4** with Human Placental Microsomes

No.	Crystallization From	Specific activity (dpm/mg) ^{3}H	^{14}C	^{3}H/^{14}C
Estriol[a]				
1	MeOH	13440	89	151
2	MeOH	13140	90	146
3	MeOH–H_2O	13200	91	145
2,4-Dibromoestriol triacetate[b]				
1	Acetone	6610	61	108
2	Acetone	6077	59	103
3	MeOH	6300	60	105

a) [1,2-^{3}H,4-^{14}C]Compound **4** (^{3}H/^{14}C=222) was incubated with human placental microsomes in the presence of NADPH at 37 °C for 2 h as described in the text. 16α-Hydroxyestrone (3 mg) was added to the extract. The steroid fraction was reduced with $NaBH_4$ and then isolated by TLC as estriol. *b*) Estriol was brominated with Br_2 in MeOH to give 2,4-dibromoestriol, which was subsequently acetylated with Ac_2O–pyridine, yielding the triacetate.

^{14}C=340) was first brominated with $CuBr_2$ to give the 16α-bromo derivative **2** which was then oxidized with 8 N CrO_3 and subsequently treated with *p*-toluenesulfonic acid, yielding the 4-ene-3-keto derivative **3**. [^{3}H, ^{14}C]Compound **3** was hydrolyzed with 1.2 eq of NaOH in aqueous pyridine to give ^{3}H- and ^{14}C-labeled compound **4** (21% from compound **1**; specific activity, ^{3}H 3.20 mCi/mmol; ^{3}H/^{14}C=222, this ratio is equivalent to a 35% loss of the tritium from DHEA which could be attributed to the C-2 position). The labeled 16α-ketol **4** was identical with authentic non-labeled compound, and its radiochemical purity was established to be more than 98% by thin-layer chromatography (TLC) and reverse isotope dilution analysis (Table I).

The distribution of tritium in the double labeled 16α-ketol **4** was initially determined by chemical and biochemical methods. After incubation of **4** with human placental microsomes

and reduced nicotinamide adenine dinucleotide phosphate (NADPH), 16α-hydroxyestrone obtained was reduced with $NaBH_4$ and then estrogen products[7] were isolated by TLC as estriol. Estriol was purified to a constant $^3H/^{14}C$ ratio by recrystallization with carrier (Table II). With this technique it was determined that 35% of the tirtium in the substrate was lost as ^3H_2O during aromatization ($^3H/^{14}C$ decreased from 222 to 145). Assuming that aromatization mechanism is completely stereospecific for the 1β,2β-hydrogen removal,[4,8] the loss of 35% tritium during conversion of the ketol **4** into estriol can be attributed to the amount of tritium located in the 1β and 2β-positions. The tritium at C-2 and C-1 in estriol corresponds to the tritium located at C-2α and C-1α of the ketol **4**. Displacement of the C-2 hydrogen would give the amount of tritium located at the C-2α position. Bromination of estriol with Br_2 in MeOH followed by acetylation with acetic anhydride in pyridine gave 2,4-dibromoestriol triacetate (the $^3H/^{14}C$ ratio decreased from 145 to 105: this is equilvalent to a 18% loss of the tritium from the labeled ketol **4**). The $^3H/^{14}C$ ratios of estriol and 2,4-dibromoestriol triacetate show that the tritium of [1,2-^{3}H, 4-^{14}C]compound **4** is distributed 47% at C-1α, 18% at C-2α, and 35% at the β-side of C-1 and C-2. However, the fact that the 2β-axial hydrogen is more labile than the 2α-hydrogen and is preferentially eliminated by the enolization of steroidal 4-en-3-ones under basic and acidic conditions[8d,9] suggests that tritium located at the

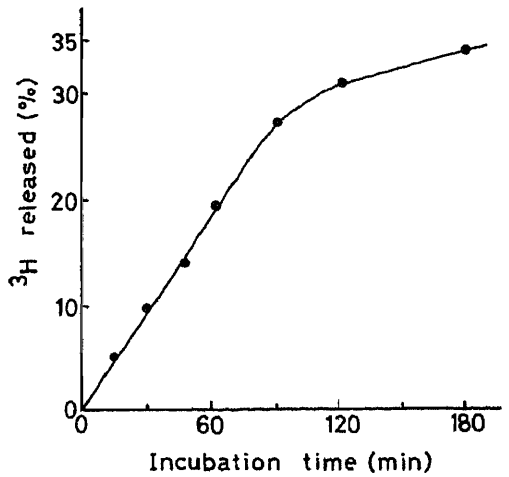

Fig. 1. Effect of Incubation Time on the ^3H_2O Formation

[1,2-^{3}H, ^{14}C]16α-Hydroxyandrostenedione (9.1 μM) was incubated with the placental microsomes (1.1 mg protein/ml) in the presence of NADPH over various time periods. The ^3H_2O formation was determined as described in the text.

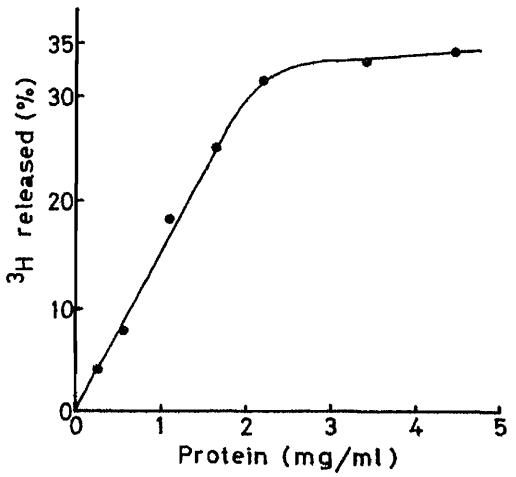

Fig. 2. Effect of Protein Concentration on the ^3H_2O Formation

[1,2-^{3}H, ^{14}C]16α-Hydroxyandrostenedione (9.1 μM) was incubated at various protein concentrations for 60 min in the presence of NADPH. The ^3H_2O formation was analyzed as described in the text.

TABLE III. Comparison of Radiometric and HPLC Methods for Determination of Aromatization of 16α-Hydroxyandrostenedione with Human Placental Microsomes

Substrate	Product	Aromatase activity pmol/mg protein/min[a]
[^{3}H,^{14}C]-Labeled	Tritiated water[b]	86±8
Non-labeled	16α-Hydroxyestrone and estriol[c]	94±7

a) Mean±S.D.; $n=4$. *b*) Radioactivity of ^3H_2O released from the labeled substrate was measured. *c*) Amounts of the products, 16α-hydroxyestrone and estriol, were determined by HPLC.

β-side would be principally incorporated at the 1β-position. The distribution at C-1 absolutely depends upon that of the commercial [1,2-^{3}H]DHEA.

To confirm that the rate of estrogen formation is equal to that of ^3H_2O formation, the [1,2-^{3}H, 4-^{14}C]16α-ketol **4** was incubated with placental microsomes in the presence of NADPH. Figure 1 shows a progress curve of the aromatization reaction as a function of time. The amount of tritium that was released and incorporated into ^3H_2O reached a plateau with 33% relative conversion to estrogens. This value is almost the same as the tritium distribution (35%) at the β-side of C-1 and C-2 of the substrate.

To further validate the ^3H_2O assay, it was necessary to establish that the rate of ^3H_2O release depends upon protein concentration. As shown in Fig. 2, the rate of aromatization of 16α-hydroxyandrostenedione as determined by measuring ^3H_2O release was proportional to protein concentration up to about 2 mg/ml. The ^3H_2O production also reached a plateau with 34% relative conversion to estrogens, and this also supports the tritium distribution at the β-side of the substrate.

The specific activity of the microsomal aromatase was then determined by employing a 60 min incubation time, 1.1 mg protein/ml, and 9.1 μM labeled substrate, which is a saturated concentration. The specific activity obtained by the radiometric assay was comparable to that obtained by the HPLC method previously developed by us[5] (Table III).

Considering the stereospecific loss of the 1β and 2β-protons of androstenedione during aromatization with the placental microsomes,[10] the tritium distribution at C-1 of the [^{3}H, ^{14}C]16-ketol **4** is not ideal for a sensitive aromatase activity determination. However, the radiometric assay using this compound is sufficiently sensitive and reliable. The obvious advantages of this method are to be found in the speed and the simplicity, and hence this quick assay should be very useful for the characterization of aromatase I in placental microsomes.

Experimental

Materials——[1,2-^{3}H(N)]Dehydroepiandrosterone (58.6 Ci/mmol) and [4-^{14}C]dehydroepiandrosterone (55 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, MA). NADPH was purchased from Sigma Chemical Co. (St. Louis, MO). 16α-Hydroxyandrostenedione and 16α-hydroxyestrone were synthesized according to a previous report.[6]

Synthesis of [1,2-^{3}H, 4-^{14}C]16α-Hydroxyandrostenedione (4)——[1,2-^{3}H]Dehydroepiandrosterone (**1**) (18 μg, 3.4 mCi) was mixed with [4-^{14}C]compound **1** (53 μg, 10 μCi) and diluted with 200 mg of non-labeled compound **1**. A solution of the ^{3}H- and ^{14}C-labeled compound **1** (200 mg, 0.69 mmol) and 2.1 mmol of $CuBr_2$ in 10 ml of dry MeOH was heated under reflux for 12 h. After the same workup as previously reported,[6] the crude bromoketone **2** (260 mg) was obtained as a solid.

A solution of the crude compound **2** (260 mg) in 50 ml of acetone was treated dropwise with 0.2 ml of 8 N CrO_3 solution with stirring below 5 °C, and then the solution was allowed to stand for 5 min. After this time, the mixture was poured into ice-water (300 ml) saturated with NaCl. The precipitates (180 mg) were collected by filtration, dried under vacuum, and then dissolved in 5 ml of acetone. *p*-Toluenesulfonic acid monohydrate (15 mg, 0.08 mmol) was added to the solution and the mixture was allowed to stand for 6 h and then poured into water (50 ml) followed by extraction with AcOEt (50 ml × 2). The organic layer was washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated to give a solid (120 mg) which was submitted to column chromatography (silica gel 6 g, hexane–AcOEt) to give a partially purified [^{3}H, ^{14}C]16α-bromo-4-androstene-3,17-dione (**3**) (95 mg).

The crude ^{3}H- and ^{14}C-labeled compound **3** (95 mg) was dissolved in 5 ml of 75% aqueous pyridine, and 0.30 ml of 1 N NaOH solution was added. The mixture was stirred at room temperature for 2 h and then poured into 50 ml of 5% HCl solution and extracted with AcOEt (50 ml × 2). The organic layer was washed with 5% HCl and 5% $NaHCO_3$ solutions and water and dried (Na_2SO_4). After evaporation of the solvent, the residue (76 mg) was submitted to column chromatography (silica gel 4 g, hexane–AcOEt) yielding the crude product **4**. Recrystallization from acetone gave **4** as colorless needles (45 mg, 21% from **1**), mp 187—188 °C (lit.[6] 188—191 °C). ^{1}H-NMR ($CDCl_3$) δ: 1.01 (1H, s, 18-Me), 1.22 (3H, s, 19-Me), 5.71 (1H, s, 4-H).

Preparation of Human Placental Microsomes——The placental microsomes were isolated from homogenates of fresh term placenta by differential centrifugation, essentially according to the method of Ryan.[11] The 105000 × *g*

(60 min) fraction was washed twice with homogenizing buffer and once with water, then freeze-dried and stored desiccated at 4 °C.

Incubation Conditions——The incubations were carried out as follows: the microsomal preparation, NADPH (3.3 μmol) and [1,2-^{3}H, 4-^{14}C]compound **4** were dissolved in MeOH (50 μl) and sufficient 0.067 M phosphate buffer (pH 7.5) to give total volume of 1.8 ml was added. The mixture was incubated at 37 °C with shaking under aerobic conditions, and terminated by addition of 4 ml of $CHCl_3$, followed by vortexing for 40 s. After centrifugation of the mixture at 2500 rpm for 10 min, aliquots (1.2 ml) were removed from the water phase and added to scintillation mixture for determination of ^3H_2O production.[4] To correct for non-specific release of tritium from the C-2 position and labeled steroids that were not extracted by $CHCl_3$, control incubations without NADPH were carried out simultaneously. The radioactivity (*ca.* 0.071% of that of the substrate) found in the aqueous phase from these blanks was subtracted from the radioactivity of ^3H_2O formed in the sample tubes.

Isolation and Bromination of Estriol——The $CHCl_3$ phases obtained from four incubation experiments using 6.1 μM labeled substrate **4** (^{3}H 1.1×10^5 dpm), and 4 mg protein of the microsomes, and incubated for 120 min incubation were combined and 3 mg of 16α-hydroxyestrone was added to the solution, then the solvent was evaporated off under an N_2 stream. $NaBH_4$ (3 mg) was added to a solution of the extract in 0.5 ml of MeOH and the mixture was kept at 0 °C for 2 h. The solvent was removed under an N_2 stream. The residue was partitioned between AcOEt (4 ml) and water (2 ml). The organic phase was washed with water, dried (Na_2SO_4) and evaporated, and the residue was submitted to TLC (silica gel 60 F_{254}, 0.5 mm of layer thickness, E. Merck; hexane–AcOEt = 1 : 4, v/v). Estriol was eluted with AcOEt. Another 20 mg of estriol was added to the eluate and the product was recrystallized from MeOH to constant specific activity (Table II).

The ^{3}H- and ^{14}C-labeled estriol (20 mg) obtained above was diluted with carrier to 20 mg and then brominated in MeOH according to the previous report[8d] to yield 2,4-dibromoestriol, mp 278—280 °C (lit.[12] 276—277 °C), which was converted into the triacetate in a usual manner, and then recrystallized to a constant specific activity (Table II).

HPLC Method for Determination of Non-labeled Compound 4 Aromatization——Estrogens, estriol and 16α-hydroxyestrone, produced by the incubation of non-labeled 16α-hydroxyandrostenedione with the placental microsomes and NADPH were determined by HPLC essentially according to the previous report.[5]

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Antitumor Action of Shiitake (*Lentinus edodes*) Fruit Bodies Orally Administered to Mice

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The powdered fruit bodies of shiitake (*Lentinus edodes*) showed antitumor activity when given orally to mice. The growth of tumors, both syngeneic and allogeneic, was inhibited by 57.9 to 78.6% in shiitake-fed mice. The degree of inhibition was proportional to the consumption of the experimental diet (L-feed) and was evident even when the administration was started from the 7th day after tumor implantation. The inhibition of tumor growth was mainly due to a glucan contained in the fruit bodies, but the lipid fracton also had inhibitory effects, although mild. Feeding of the fruit bodies to mice in the diet was found to augment both the ability of macrophages to phagocytose latex particles and the spreading activity of the macrophages.

Keywords——shiitake (*Lentinus edodes*); oral administration; antitumor activity; phagocytosis

Chihara *et al.*[1] recently, isolated a β-1,3 glucan from *Lentinus edodes* (shiitake). It is made up of a β-1,3 glucopyranoside main chain with β-1,6 glucopyranoside chains branched at rate of two per five glucose units (lentinan). Small doses of this β-glucan were shown to produce complete regression of various allogeneic and syngeneic tumor implants in mice.[2,3] When used in combination with chemotherapy, they prolonged the survival of the mice bearing autologous tumors.[4] However, all the above antitumor effects were obtained when mice were repeatedly treated either intraperitoneally or subcutaneously with the purified lentinan. The fruit bodies containing lentinan were given orally in this study so as to determine whether or not they have antitumor effects on allogeneic or syngeneic tumors after oral administration.

Materials and Methods

Animals——Four- to 5-week-old male ICR and 5- to 6-week-old C3H/He, BALB/C, C57BL/6 CDF_1 mice were obtained from Charles River Japan Inc.

Assay for Antitumor Activity——Sarcoma-180, ascites from, was maintained in the peritoneal cavity of ICR mice by weekly transplantation. Syngeneic IMC carcinoma cells were transplanted into CDF_1 mice (6 weeks), MM-46 carcinoma into male C3H mice (6 weeks), Meth-A fibrosarcoma into male BALB/C mice (7 weeks) and B-16 melanoma and Lewis lung carcinoma into C57BL/6 mice (6 weeks). In all experiments, tumor cells were collected from the peritoneal cavity of tumor-bearing male mice and 2×10^6 cells were implanted subcutanously in the right axillary region of male mice. On the 25th day after tumor transplantation, the solid tumor was extirpated and weighed to obtain the tumor growth inhibition rate, which was evaluated as: [1 − (weight of tumor mass from animals treated with L-feed ÷ weight of tumor mass from animals receiving no treatment] × 100. In the case of the ascites tumors, male mice which had received 2×10^6 tumor cells intraperitoneally were given powdered shiitake fruit bodies in their diet and their longevity was evaluated in terms of the time (d) required for a half of the animals to die (median survival %).

Preparation of Feeds Containing Powdered Shiitake Fruit Bodies (L-Feed)——The fruit bodies, produced at the

Mushroom Research Institute of Japan (Kiryu, Gumma), were dried and pulverized into fine pieces (ϕ 0.05 nm). They were mixed with commercial mouse feed (CRF-1, Charles River, Japan, Inc.) at 10, 20 and 30%. Each mixture was kneaded into a paste with distilled water. The paste was cut into pieces (5 × 2 × 3 cm) and dried at 60—80 °C for 2—3 d. The completely dried feeds were used as 10% L-feed, 20% L-feed and 30% L-feed.

Measurement of Spreading Rate[5]——Macrophages were obtained by washing with Hanks' solution the peritoneal cavity of mice fed the L-feed for 7—12 d. The cells were suspended in RPMI-1640 medium and 1×10^6 cells per well were inoculated onto a 24-well plastic plate (Linbro Flow, Lab. U.S.A.). The plate was incubated at 35 °C in a 5% CO_2 atmosphere and non-adherent cells were removed by washing 3 times with Hanks' solution. The macrophages were incubated with 1 ml of RPMI-1640 medium in the same manner as above. The cells were fixed for 2 h with 2.5% glutaraldehyde and washed more 3 times in MeOH, then stained with Giemsa. The number of spreading cells was determined by examining 200 cells per sample at random.

Phagocytic Assay[6]——Macrophages were obtained from the peritoneal cavity of mice maintained on L-feed for 7—12 d, and 1×10^7 cells were cultured in a medium (RPMI-1640) containing latex particles (fluoresbrite carboxylate microspheres, DIA = 2μ, SD = 0.03, Polysciences, Inc., Warrington) for 60 min. The extra-cellular latex particles were rinsed away with phosphate-buffered saline. The number of intra-cellular latex particles per 200 cells was counted to assess the phagocytic activity.

Results and Discussion

According to Chihara *et al.*,[2] the β-glucan (lentinan) extracted from shiitake inhibited the growth of various tumors by more than 90% in mice when it was injected intraperitoneally once a day for 10 d. However, no report to date deals with the antitumor effect of oral administration of shiitake fruit bodies.

Figure 1 illustrates the inhibitory effects on tumor growth in mice receiving the experimental diet prepared from commercial mouse feed and shiitake powder. Female 5-week-old ICR mice, into which 2×10^6 Sarcoma-180 cells had been implanted, were maintained on feeds containing powdered shiitake for 25 d. The tumor growth was inhibited

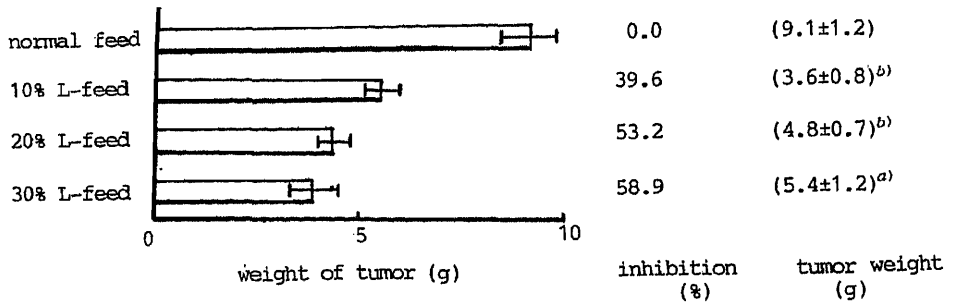


Fig. 1. Effects of Shiitake Powder on Growth of Sarcoma-180

Significance of differences (*t*-test): *a*) $p < 0.05$, *b*) $p < 0.01$.

TABLE I. Antitumor Effects of L-Feed against Various Syngeneic Tumors

Tumor	Solid type	Ascites type
	Inhibition (%)	Median survival (%)[a]
B-16 melanoma in C57BL/6N mouse	11.3±0.31[b]	−1.9±0.5
Lewis lung carcinoma in C57BL/6N mouse	23.7±0.52[c]	17.1±0.7[b]
IMC carcinoma in CDF_1 mouse	21.3±1.1[b]	9.3±1.1
Meth-A fibrosarcoma in BALB/C mouse	4.3±1.8	5.1±1.4
MM-46 carcinoma in C3H mouse	78.6±0.72[d]	25.5±0.7[b]

a) Increase of life span (ILS) in terms of median survival (%). Significance of differences (*t*-test): *b*) $p < 0.05$, *c*) $p < 0.01$, *d*) $p < 0.001$ (9 mice × 3).

by about 40, 53 and 58% by 10, 20 and 30% L-feed, respectively. Thus, the inhibitory activity increased with the concentration of the shiitake powder. On the basis of these results, 20% L-feed was used in the subsequent experiments. The antitumor effects of the 20% L-feed on the growth of syngeneic tumors are summarized in Table I.

The dietary administration had essentially no effect on the growth of solid or ascites tumors of B-16 melanoma, Lewis lung carcinoma, meth-A fibrosarcoma and IMC carcinoma, but inhibited the growth of solid MM-46 carcinoma in C3H mice by about 79%. These results indicate that L-feed can inhibit the growth of some syngeneic tumors as well as allogeneic ones.

The powdered shiitake fruit bodies (A) were separated to 4 fractions, B, C, D and E. The antitumor activity of each fraction was assessed in Sarcoma-180 tumor-bearing ICR mice. The powdered shiitake fruit bodies (A) were extracted for 8 h at 42 °C with a 1 : 1 mixture of EtOEt and EtOH. The extraction was repeated 3 times. The extracts obtained were combined and evaporated under N_2 gas. The extractable material (lipid) was taken as B fraction. The residue of shiitake powder was extracted with EtOEt and EtOH mixture to provide C fraction. The insoluble residue of the extraction (lipid) was treated with hot water at 120 °C for 2 h for the extraction of glucan. The residue was taken as E fraction. The shiitake powder (A) which had been treated with hot water was filtered to exclude soluble glucan and the residue was taken as fraction D. Allogeneic Sarcoma-180 tumor-bearing ICR mice were fed with the experimental diet containing A or one of the B, C, D and E fractions for 25 d. Figure

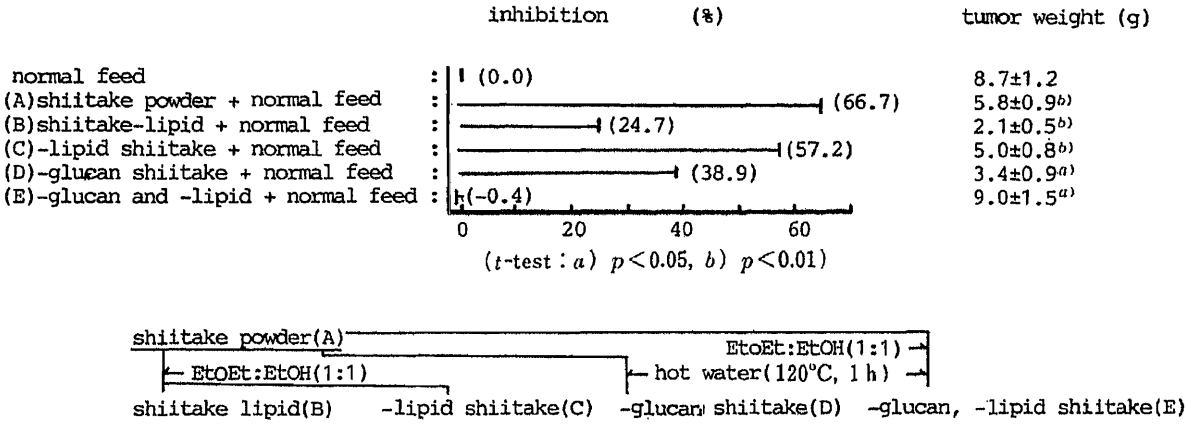
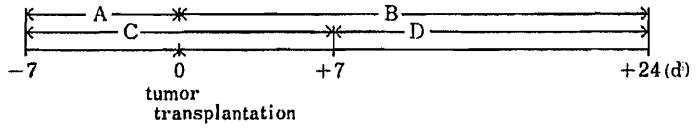


Fig. 2. Inhibitory Effects of Various Fractions of Shiitake on the Growth of Solid-Type Sarcoma-180 in ICR Mice (8 Mice × 3)

TABLE II. Effects of L-Feed on the Growth of Sarcoma-180 in ICR Mice

	Schedule of feedings	Tumor weight (g)	TIR (%)
(1)	A (normal feed→7 d) ——→B (normal feed→24 d)	4.75±0.88	0.0
(2)	A (L-feed→7 d) ——→B (normal feed→24 d)	4.67±1.14	1.7
(3)	A (normal feed→7 d) ——→B (L-feed→24 d)	1.31±0.98b)	72.4
(4)	C (normal feed→14 d) ——→D (L-feed→17 d)	2.19±1.47a)	53.9

Significance of differences (*t*-test): *a*) $p<0.05$, *b*) $p<0.01$ (14 mice × 2). TIR:
Scheme of feeding schedules:



2 indicates the inhibitory effects on tumor growth. Fraction A, the whole powder of fruit bodies, inhibited the growth by 66.7%. When lipid was excluded from A, the rate decreased to 57.2%. The antitumor activity was totally lost when both lipid and glucan were removed from A as fraction E. When tumor-bearing mice received the lipid fraction in the diet, the growth of tumors was inhibited by 24.7%. These results suggest that the antitumor effect of L-feed is due mainly to constituents soluble in hot water (lentinan), and that the lipid fraction also has antitumor activity, although mild. The composition of the lipid extracted with EtOEt–EtOH mixture has not yet been clarified.

Next, the influence of administration schedule of the experimental L-feed relative to tumor implantation was examined. The results are shown in Table II. In the case of pretreated mice (schedule 2), the growth of tumors was not inhibited at all. However, the growth of the tumors was inhibited by 72.4% when feeding of the L-feed was started immediately after tumor implantation (schedule 3). In mice given L-feed only from 7 d after implantation (when the tumors were 2—3 mm in diameter), the growth inhibition amounted to 53.9%, indicating quite potent antitumor activity. These results indicated that the fruit bodies have inhibitory effects on the growth of tumors only when they are ingested after implantation of tumor cells. The ingestion is prophylactically ineffective.

According to Chihara *et al.*,[2] lentinan contained in the fruit bodies of shiitake acts as an immunostimulant to develop tumor immunity. Thus, in order to study the mechanism of the antitumor action of powdered fruit bodies following oral administration, the spreading and phagocytic activities of intraperitoneal macrophages obtained from tumor-bearing mice were studied. For these studies two host-tumor model systems in which the dietary administration had 78.6% and 21.3% inhibitory effects, MM-46 carcinoma-bearing C3H mice and IMC carcinoma-bearing CDF_1 mice, respectively, were used. The effects on spreading rate, which is considered to reflect the activation of macrophages, are summarized in Table III.

Table III. Spreading Rate of Macrophages

Feed	Rate[a]			
	CDF_1-mice		C3H-mice	
	7 d	12 d	7 d	12 d[b]
Normal feed (control)	1.00	1.00	1.00	1.00
L-Feed	0.88 ± 0.05[c]	0.86 ± 0.03[d]	2.03 ± 0.08[d]	3.65 ± 0.09[c]

a) Spreading rate = No. of spreading cells/total cells. *b*) Feeding time. Significance of differences (*t*-test): *c*) $p < 0.05$, *d*) $p < 0.01$.

Table IV. Phagocytosis of Latex Particles by Macrophages

Feed	Phagocytic enhancement ratio			
	CDF_1 mice		C3H mice	
	7 d	12 d[a]	7 d	12 d[a]
Normal feed (control)	1.00	1.00	1.00	1.00
L-Feed	0.67 ± 0.06[b]	1.25 ± 0.08[b]	1.08 ± 0.2	1.79 ± 0.05[b]

a) Feeding time. Significance of differences (*t*-test): *b*) $p < 0.05$ (5 mice × 4).

Because activated macrophages adhere to glass and expand to take a special form, they are easily distinguished from non-activated ones. The spreading rate of macrophages from normal CDF_1 mice was decreased a little when the mice were fed on the L-feed, but the rates for macrophages from normal C3H mice given the L-feed for 7 and 12 d increased 2.0 and 3.65 times, respectively. As shown in Table IV, L-feed for 7 d did not increase phagocytic activity, as measured with latex particles. However, the phagocytic activity of macrophages from normal C3H mice increased about 1.8 times when the mice were fed on the L-feed for 12 d.

In general, cellular immunity including that involving macrophages has been reported to decline in tumor-bearing animals. Therefore, the influence of shiitake powder on macrophages from tumor-bearing mice was studied. As shown in Table V, when CDF_1 mice were given normal feed, the spreading rate of macrophages from tumor-bearing mice was 51—46% of that for normal mice, but the L-feed elevated it to 74%. In C3H mice implanted with MM-46 tumor cells, the spreading rate was decreased to 63—42% with normal feed, but the L-feed restored the rate to the normal level in association with a remarkable inhibition of the tumor growth.

Table VI shows the phagocytic activity of the macrophages, as examined with latex particles. In tumor-bearing CDF_1 mice given normal feed, the phagocytic activity decreased to as little as 37—31% of the controls, but the L-feed raised the level to 67—89%. In MM-46 carcinoma-bearing C3H mice, the activity was decreased to 48—39% of that for normal mice, but recovered to its normal level in mice given the L-feed for 7 d. When the L-feed was given for 12 d, the activity was elevated beyond the normal level (to 118%). These facts indicate that the orally administered fruit bodies of shiitake are able to inhibit the growth not only of allogeneic tumors, but also of syngeneic tumors such as MM-46 carcinoma, as can

Table V. Spreading Rate of Macrophages from Tumor-Bearing Mice

Feed	State	Spreading rate			
		IMC in CDF_1		MM-46 in C3H	
		7 d	12 d[a]	7 d	12 d[a]
Normal feed (control)	Non-tumor bearing	1.00	1.00	1.00	1.00
	Tumor-bearing	0.51 ± 0.03^{b}	0.46 ± 0.02^{b}	0.63 ± 0.04^{b}	0.42 ± 0.09
L-Feed	Tumor-bearing	0.74 ± 0.02^{c}	0.77 ± 0.02^{c}	1.05 ± 0.8	1.08 ± 0.02^{b}

a) Feeding times after tumor implantation. Significance of differences (*t*-test): *b*) $p<0.05$, *c*) $p<0.01$ (6 mice × 3).

Table VI. Pagocytosis of Latex Particles by Macrophages from Tumor-Bearing Mice

Feed	State	Spreading rate			
		IMC in CDF_1		MM-46 in C3H	
		7 d	12 d[a]	7 d	12 d[a]
Normal feed (control)	Non-tumor bearing	1.00	1.00	1.00	1.00
	Tumor-bearing	0.37 ± 0.02^{b}	0.31 ± 0.09	0.48 ± 0.01^{c}	0.39 ± 0.02^{b}
L-Feed	Tumor-bearing	0.67 ± 0.03^{b}	0.89 ± 0.02^{c}	0.97 ± 0.03^{b}	1.18 ± 0.04^{b}

a) Feeding times after implantation. Significance of differences (*t*-test): *b*) $p<0.05$, *c*) $p<0.01$ (7 mice × 4).

intraperitoneal doses of lentinan, which was extracted from shiitake. The antitumor activity increased with the amount of the fruit bodies administered. These results suggest that the tumor inhibition might be based on potentiation of the phagocytic activity of macrophages, since this is depressed in tumor-bearing animals. The effects of the shiitake powder on other immunological functions will described in subsequent reports.

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Antitumor Mechanisms of Orally Administered Shiitake Fruit Bodies

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When tumor-bearing mice were given diet containing fruit bodies of shiitake (L-feed), the tumor growth was apparently inhibited. To elucidate the mechanisms of this action, the effects of the L-feed on the production superoxide anion (SOA) by macrophages and the cytotoxicity of natural killer T cells were studied. When MM-46 carcinoma-bearing C3H mice were maintained on L-feed (containing 20% shiitake powder), SOA production by macrophages was increased about 2.0 to 2.3 times. Furthermore, the cytotoxic activities of natural killer cells and killer T cells were increased 1.9 times and 1.4 times, compared with their counterparts from normal C3H mice fed on shiitake-free diet. These results suggest that shiitake powder given orally activates various effector cells to attack tumor cells. It appears to potentiate cellular functions and at the same time to prevent a decrease of immune function of the tumor-bearing host.

Keywords——shiitake; antitumor activity; macrophage; natural killer cell; superoxide anion; cytotoxic T cell

Dietary administration of shiitake (*L. edodes*) fruit bodies was shown previously to inhibit the growth of both allogeneic and syngeneic tumors in mice.[1] This antitumor activity was suggested to depend on immunological effector cells including macrophages. To delineate the mechanism further, the present study was designed. According to Chihara *et al.*,[2] lentinan, a glucan consisting of a β-1,3-glucopyranoside chain with 1,6-glucopyranoside branches, extracted from the fruit bodies of shiitake acts as an immunostimulant to develop tumor immunity against allogeneic and some syngeneic tumors. It is widely accepted that activated macrophages, cytotoxic T cells, natural killer cells, and killer T cells usually play important roles in tumor immunity.[2,3] Lentinan has been reported to enhance the activity of these immune systems when administered intraperitoneally, subcutaneously, or intravenously. In order to study the mechanisms of antitumor action following oral administration, mice were maintained on feed containing powdered shiitake fruit bodies (L-feed) and the effects on the activities of macrophages and cytotoxic T cells were examined. The results are described in this paper.

Materials and Methods

Feeds——The experimental diet containing 20% powdered shiitake fruit bodies, which were provided by the Mushroom Research Institute of Japan (Kiryu, Gumma), were prepared as described previously (L-feed).[1] Mice receiving shiitake-free diet (normal feed) served as controls.

Animals——Male mice of the ICR strain (5 to 6 weeks old), of the C3H strain (6 weeks old), and of the CDF_1 strain (5 to 6 weeks old) were obtained from Charles River Japan, and were maintained on commercial mouse chow for 1 week then used for the experiment.

Collection of Macrophages——The CDF_1 and C3H mice were maintained on either normal feed or L-feed during the periods indicated, and their peritoneal cavities were rinsed with Hanks' solution. The solution was centrifuged at 1200 rpm for 10 min to collect cells. The cell pellet was suspended in RPMI-1640 medium, and the suspension was adjusted to 1×10^6 cells. Then 1.5 ml portions of this suspension were seeded onto a plastic plate, and the plate was incubated at 37 °C for 2 h in an atmosphere of 5% CO_2 in air saturated with water vapor. Macrophages adhered to

the plastic plate. Non-adherent cells were removed by rinsing the plate 3 times with Hanks' solution.

Preparation of Fluorescen-*Listeria*——*Listeria* were inoculated into 10 ml of nutrient broth medium containing 0.1 ml of fluorescen solution and cultured at 37 °C for 24—36 h. The culture was centrifuged at 1200 rpm for 10 min. The bacterial sediment was washed 3 times with the same medium and heated at 100 °C for 10 min, then suspended in saline for the experiment. The amount of *Listeria* in phagosomes of macrophages was measured by flow cytometry (Ortho Spectrum III).

Determination of Superoxide Anion (SOA)——Male mice of the ICR strain (5 to 6 weeks old), of the C3H strain (6 to 7 weeks old), and of the CDF_1 strain (6 to 7 weeks old) were maintained on the normal feed or the L-feed. Peritoneal macrophages were obtained from the mice as described above, and the amount of released SOA was measured by the method of Ito *et al.*[4] A 1.5 ml aliquot of phosphate-buffered saline (PBS) containing 10 mM glucose, 80 μM ferricytochrome c, and 0.2 mg/ml opsonized zymosan was added to macrophages adhering to a plate. The mixture was incubated for 90 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred into a test tube chilled with ice to stop the reaction and the absorbance was measured at 550 nm. A 1 ml aliquot of 0.5% sodium dodecyl sulfate (SDS) was added to the cells remaining at the bottom of the plate. After 5 min, the number of cells in the solution was determined by measuring protein content according to Lowry's method.[5] The quantity (nmol) of ferricytochrome c was obtained from the absorbance at 550 nm, according to the formula ΔE 550 nm $=2.1 \times 10^4 \text{M}^{-1}$, and the amount of SOA per unit protein was calculated. For opsonization of zymosan, zymosan A was adjusted with PBS to 50 mg/ml, boiled for 1 h, washed 3 times, and resuspended in PBS at 50 mg/ml. Four volumes of human serum and one volume of 50 mg/ml zymosan were incubated at 37 °C for 30 min and after centrifugation, 10 mg of opsonized zymosan was resuspended in 1 ml of PBS.

Phagocytic Activity Assay——MM-46 carcinoma tumor cells, preliminarily cultured for 7 d in the peritoneal cavity of C3H mice, were collected and washed with Hanks' solution by centrifugation (1200 rpm, 5 min), and 2×10^7 tumor cells were resuspended in 1 ml of RPMI-1640 medium containing 1.0 μCi (25 μl) of ^{3}H-uridine. The suspension was cultured for 2 h, then free ^{3}H-uridine was rinsed away with about 500 ml of Hanks' solution.[6] The labeled tumor cells were submitted to phagocytic assay at an E/T ratio of 50 : 1 for 2 h. After incubation, adherent macrophages were collected and dissolved in toluene. The radioactivity (dpm) incorporated into macrophages was counted on a liquid scintillation counter (Aloka LSC-700). The presence of phagocytosed tumor cells in macrophages was detected by microscopy.

Preparation of Spleen Cell Suspension[7]——A male C3H mouse (6 to 7 weeks old) was killed by vertebral dislocation. The mouse was bled by cutting the femoral vein and the spleen was extirpated. After being washed with Eagle's minimum essential medium (MEM), the spleen was teased with scissors and passed through an 80 mesh stainless steel sieve. The cells were collected by centrifugation at 1200 rpm for 10 min, than 2 ml of 10-fold dilution of Eagle's MEM was added to lyse contaminating erythrocytes hypotonically for 10 s. The cell suspension was immediately added to 2 ml of Eagle's MEM (2-fold concentration). The suspension was centrifuged at 1200 rpm for 10 min, and the cells thus obtained were adjusted to 1×10^7 cells/ml with RPMI-1640 medium. This was used as the whole spleen cell suspension. This suspension was placed in a Petri dish (5 cm in diameter) and incubated for 60 min. Then, non-adherent cells were exclusively collected, adjusted to 1×10^7 cells/ml with RPMI-1640 medium, and used as non-adherent spleen cells. In this experiment, the cytotoxic activity of NK was assayed at an E/T ratio of 50 : 1.

Elimination of T Cells[8]——A mixture of 1 ml of non-adherent spleen cell suspension obtained from male C3H mice (7 weeks) and 20 μl of 5% Thy-1.2 F7D5 monoclonal immunoglobulin M antibody (Serotec Ltd. England) was incubated for 30 min, then the cells were washed once with RPMI-1640 medium. After that, 1 ml of RPMI-1640 medium with fetal bovine serum containing 5% guinea pig complement was added, and the whole was further incubated for 30 min. The cells that reacted with antibody were selectively destroyed. The remaining cells were sedimented by centrifugation at 1200 rpm for 10 min and rinsed 3 times with RPMI-1640 medium. The cells were adjusted to 1×10^7 cells/ml.

Cytotoxicity——A 1 ml aliquot of the labeled target cell suspension (2×10^5 cells/ml) was mixed with 1 ml of the lymphocyte suspension (1×10^7 cells/ml) and 0.5 ml aliquots were incubated for 4 h. Then, 0.4 ml of reaction mixture was suction-filtered through a Millipore filter (0.45 nm pore size, Millipore Co.). After being washed with 15 ml of cold 5% trichloroacetic acid (TCA) solution, the filter was dried and mixed with 10 ml of lipophilic scintillator. Radioactivity was measured with a scintillation counter. The cytotoxicity (P%) was calculated according to the following formula:

$$P(\%)=\left(1-\frac{\text{dpm with immunolymphoyctes-background dpm}}{\text{dpm when target cells only were incubated-background dpm}}\right)\times 100$$

Results and Discussion

In the previous paper,[1] we reported that the inhibitory effect of shiitake fruit bodies on the growth of tumors varied depending on the type of tumor–host system. MM-46 carcinoma

in C3H mouse showed about 80% tumor growth inhibition, whereas with IMC carcinoma in CDF_1 mouse the inhibition was as low as about 20%. Therefore, these two systems were chosen for this study. At 10 d after tumor implantation, peritoneal macrophages were collected and assayed for phagocytic activity towards fluorescen-labeled *Listeria*. As shown in Table I, the phagocytic activity of macrophages from tumor-bearing C3H mice increased 2.3 times, when the hosts had been given L-feed. However, the activity was decreased when they had been maintained on normal feed. In the case of tumor-bearing CDF_1 mice, the activity was increased about 1.8 times.

The phagocytic activity of macrophages against syngeneic tumor cells was examined. As shown in Fig. 1, macrophages from mice given normal feed showed no alteration of phagocytic activity against MM-46 tumor cells, regardless of duration of feeding. On the other hand, when mice were maintained on L-feed, the phagocytic ability was increased with duration of feeding. The number of tumor cells phagocytosed by macrophages reached about 18—25% of total tumor cells used.

The study was centered on the amount of SOA released by macrophage in phagocytosis, as one of the criteria of cytotoxicity of activated macrophages. As shown in Table II, macrophages obtained from ICR mice given L-feed for 15 d after implantation of Sarcoma-

Table I. Phagocytic Activities of Macrophages from CDF_1 and C3H Mice Given L-Feed or Normal Feed

Mice and tumor		Feed	No. of *Listeria*[a]	Ratio
C3H mice	Non-bearing	Normal feed	17.1±2.2	1.00
		L-Feed	31.2±1.1[c]	1.83
	MM-46 bearing	Normal feed	12.6±1.3[b]	0.74
		L-Feed	39.3±1.1[d]	2.31
CDF_1 mice	Non-bearing	Normal feed	13.2±3.2	1.00
		L-Feed	15.5±0.8[b]	1.17
	IMC-bearing	Normal feed	8.2±0.5[c]	0.62
		L-Feed	23.4±1.3[c]	1.77

a) No. of *Listeria*/cell (average of 200 cells). Significance of differences (*t*-test): *b*) $p<0.05$, *c*) $p<0.01$, *d*) $p<0.001$.

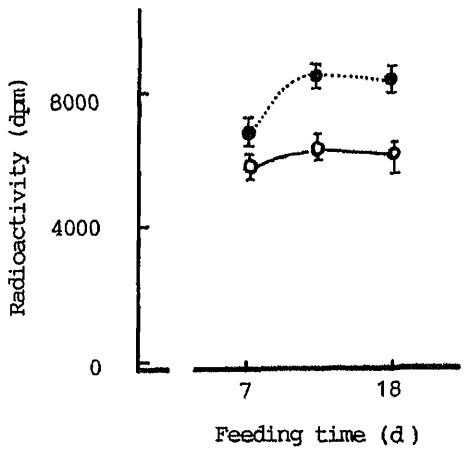

Fig. 1. Phagocytic Activity of ^{3}H-Uridine-Labeled MM-46 Carcinoma by Macrophages from C3H Mice Given L-Feed or Normal Feed

○—○, normal feed; ●---●, L-feed.

Table II. Release of SOA by L-Feed-Induced Macrophages from Tumor-Bearing Mice

Mice	Feed	SOA release (nmol/mg protein)	Ratio
Normal ICR	Normal feed	12.40±0.83	1.00
	L-Feed	13.65±1.13	1.10
Sarcoma-180 bearing ICR	Normal feed	11.83±0.78[a]	0.95
	L-Feed	25.86±1.06[b]	2.08

Significance of differences (*t*-test): *a*) $p<0.05$, *b*) $p<0.01$.

TABLE III. Time Course of SOA Release by L-Feed-Induced Peritoneal Macrophage from IMC Tumor-Bearing Mice

Mice and tumor	Feed	SOA release					
		After 10 d			After 20 d		
		(nmol/mg protein)	(ratio)		(nmol/mg protein)	(ratio)	
Normal CDF_1	Normal feed	7.8±2.42	(1.00)		4.8±1.83	(1.00)	
	L-Feed	9.2±1.52[a)]	(1.18)		8.6±1.73[a)]	(1.79)	
IMC-bearing CDF_1	Normal feed	6.2±3.11	(0.79)	(1.00)	3.4±1.97	(0.70)	(1.00)
	L-Feed	8.3±1.11[a)]	(1.06)	(1.34)	6.6±1.95[a)]	(1.37)	(1.94)

Significance of differences (*t*-test): *a*) $p<0.05$.

TABLE IV. Time Course of SOA Release by L-Feed-Induced Peritoneal Macrophages from MM-46 Tumor-Bearing Mice

Mice and tumor	Feed	SOA release					
		After 9 d			After 18 d		
		(nmol/mg protein)	(ratio)		(nmol/mg protein)	(ratio)	
Normal C3H	Normal feed	26.9±0.87	(1.00)		18.9±1.08	(1.00)	
	L-Feed	25.3±0.79	(0.94)		20.1±0.77	(1.06)	
MM-46 bearing C3H	Normal feed	19.9±0.97	(0.74)	(1.00)	9.5±1.43	(0.50)	(1.00)
	L-Feed	40.1±0.85[a)]	(1.49)	(2.01)	21.9±1.18[a)]	(1.16)	(2.31)

Significance of differences (*t*-test): *a*) $p<0.05$.

180 tumor cells produced less SOA than those from their tumor-free counterparts. When the mice were given L-feed, macrophages obtained from tumor-bearing mice released twice as much SOA as those from normal mice.

The results on SOA production in macrophages obtained from syngeneic tumor-bearing mice are summarized in Tables III and IV. In the case of CDF_1 mice given L-feed for 10 or 20 d after implantation of IMC-tumor cells, the SOA production was increased to 1.1—1.4 times that of normal mice given normal feed. However, as shown in Table IV, in the case of C3H mice (in which the inhibition of MM-46 tumor growth was greater), the amount of released SOA was increased about 2.0—2.3 times by L-feed for 9 d. However, when the mice were given L-feed for 18 d, the produced SOA was almost the same as that for normal mice given normal feed for 18 d.

These results suggest that tumor growth inhibition by the shiitake fruit bodies is at least partly due to enhanced production of SOA by macrophages or prevention of deterioration of immuno-phagocytosis in the tumor-bearing state.

Next, the effects of shiitake powder on NK cells (nonspecifically injuring tumor cells), and cytotoxic T cells (Tc, specifically injuring antigen-presenting cells) were studied. First of all, NK cells, which are induced from pre-NK cells by IFN, were studied. Spleen cells were used as a cell group containing NK cells, and YAC-1 tumor cells were labeled with ^{3}H-uridine as target cells. C3H mice were given L-feed for 8 d, and non-adherent spleen cell suspension was prepared from the extirpated spleen. The cytotoxicity of this cell fraction against YAC-1 tumor cells was measured at an E/T ratio of 50 : 1. As shown in Table V, the NK cell activity

TABLE V. Effects of L-Feed on NK Activity of Non-adherent Spleen Lymphocytes against YAC-1 Tumor Cells

Feed	NK activity (%) (relative cytotoxicity)	Ratio
Normal feed	6.83±1.79	1.00
L-Feed	12.84±0.88[a)]	1.88

Significance of difference (*t*-test): *a*) $p<0.05$. E/T (NK cell 1×10^7: YAC-1 cell 2×10^5) ratio=50:1.

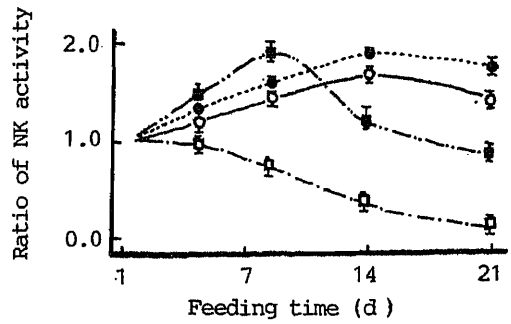

Fig. 2. Time Course of Activities of NK Cells from C3H Mice Given L-Feed or Normal Feed

○—○, normal C3H (normal feed); ●---●, normal C3H (L-feed); □---□, MM-46-bearing (normal feed); ■---■, MM-46-bearing (L-feed).

TABLE VI. Effects of L-Feed on the Development of Allogeneic Cytotoxic T-Lymphocytes

Feed	Treatment	Cytolysis (%)	Ratio	
Normal feed	Non	21.4±0.63	1.00	1.00
	Anti-Thy1.2+C′	11.5±1.52	0.54	
L-Feed	Non	30.7±1.39[b)]	1.00	1.43
	Anti-Thy1.2+C′	14.5±0.99[a)]	0.47	

Significance of differences (*t*-test): *a*) $p<0.05$, *b*) $p<0.01$.

was potentiated about 1.9 times by the L-feed, compared with that for the same cell fraction from mice given normal feed.

MM-46 tumor-bearing C3H mice were maintained on L-feed, and spleen cells containing NK cells were obtained from the isolated spleen. The cytotoxic activity against P-815 tumor cells was assessed. The cytotoxicity in tumor-bearing mice might be due in part to lymphokine-activated killer cells (LAK), but the whole cytotoxic activity including both NK and LAK was taken as activity of NK cells in this experiment, because the separation of NK cells from the other cells was difficult. As shown in Fig. 2, in the case of tumor-bearing mice, the cytotoxicity of NK cells were clearly decreased, but after L-feed for 7 d, the activity rose transiently to above the normal level. After that, the activity decreased, but this reduction was slower than that in mice receiving normal feed. When the tumor-bearing mice were given L-feed for 21 d, the cytotoxic activity of NK cell was about 71% of the level of normal mice. However, the activity was only 21% when the tumor-bearing mice were given normal feed for the same time. These results suggested that shiitake powder directly potentiates the cytotoxicity of NK cells, or accelerates the differentiation of inactive pre-NK cells to active NK cells.

Generally, T cells (Tc) show specific cytotoxicity against allogeneic cells used for immunization. According to Hamuro *et al.*,[9] β-glucans including lentinan potentiate mouse Tc induction *in vivo* and *in vitro*. Whether shiitake fruit bodies (containing lentinan) show a similar effect or not was studied. The results are given in Table VI. After sensitization by grafting P-815 tumor cells as antigen cells intraperitoneally into C3H mice, mice were given L-feed for 15 d. Then, their spleens were extirpated, and suspensions of whole spleen cells and spleen cells containing no T cells were prepared. The cytotoxicity of whole spleen cell suspension was increased 1.4 times by shiitake powder, but the fractions lacking T cells

showed a marked decrease. The results suggest that the cytotoxicity potentiated by oral administration of shiitake fruit bodies is that of T cells. That is, shiitake fruit bodies accelerate the induction of Tc cells.

In summary, the action mechanisms of orally administered fruit bodies of shiitake seem to be similar to those of lentinan injected i.p. into mice. Shiitake powder showed antitumor action against several syngeneic tumors, and acts not only by activation of various effector cells (Mø, NK cells, Tc, *etc.*) to attack tumor cells, but also by potentiating the cellular functions and preventing a decrease of immune functions of the tumor-bearing host.

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Renal Effect of Aqueous Extract from Salviae miltiorrhizae Radix in Normal Rats

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The renal effect of Salviae miltiorrhizae Radix extract was examined in normal rats. A single intraperitoneal administration of the extract led to appreciable increments of urine volume, and urinary excretion of urea and creatinine. The extract also induced natriuretic, kaliuretic, and phosphaturic responses. Furthermore, treatment with Salviae miltiorrhizae Radix significantly increased glomerular filtration rate, renal plasma flow, and renal blood flow.

Keywords——Salviae miltiorrhizae Radix; urea; creatinine; electrolyte; glomerular filtration rate; renal plasma flow; renal blood flow; rat

In Chinese medical literature, it is recorded that Salviae miltiorrhizae Radix improves blood circulation, relieves blood stasis, eliminates swellings, regulates menstruation, *etc.*[1] On the other hand, it has recently been reported to show vasodilative, hypotensive, anticoagulant, and antibacterial activities and to have a beneficial effect in patients with chronic renal failure by many clinical and laboratory researchers.[2,3] However, there are few reports on the effects of Salviae miltiorrhizae Radix on renal function and urinary excretion of nitrogen compounds, electrolytes, and so forth. Therefore, in our laboratory, a series of studies on the effects of Salviae miltiorrhizae Radix extract has been carried out.

As reported previously, the chronic administration of Salviae miltiorrhizae Radix extract to rats with uremia induced by an adenine diet resulted in significant decreases of uremic toxins such as urea nitrogen, creatinine, methylguanidine, and guanidinosuccinic acid in the serum.[4-6] An increase in renal tissue blood flow together with a fall of blood pressure during Salviae miltiorrhizae Radix extract administration was observed, which suggested that the extract might alleviate the uremia by enhancing renal function.[7]

In the present paper, further studies were carried out to investigate the effects of Salviae miltiorrhizae Radix extract on the urinary excretion of urea, creatinine, and electrolytes and on the parameters of renal function, *i.e.*, glomerular filtration rate (GFR), renal plasma flow (RPF), and renal blood flow (RBF). Specifically, these studies were designed to focus on the renal effects after an intraperitoneal single dose of Salviae miltiorrhizae Radix extract in normal rats, to establish how this extract affects renal function.

Materials and Methods

Animals and Treatments——Male rats of the LWH: Wistar strain, with a body weight of 200—210 g, were placed in metabolic cages at a temperature of 25±1 °C with a 12 h dark-light cycle. They were allowed an adaptation period of 10 d and fed on a commercial feed (CLEA Japan Inc., Tokyo, Japan, type CE-2) during the adaptation period. Then the aqueous extract from Salviae miltiorrhizae Radix (10 mg/100 g of body weight) in saline was administered intraperitoneally to the rats, while control rats received an equal volume of saline. Throughout the experimental period, they were deprived of food and received tap water *ad libitum*. There was no significant difference between the

control and the extract-treated rats with regard to water intake.

Preparation of Salviae miltiorrhizae Radix Extract——The roots of Salviae miltiorrhizae Radix (*Salvia miltiorrhiza* BUNGE) produced in China, supplied by Tochimoto Tenkaido Co., Ltd., Osaka, Japan, were finely powdered and extracted with distilled water at 100 °C for 40 min (roots : water = 1 : 10, w/v), as described previously.[4] The aqueous extract was filtered through 4 layers of gauze and the filtrate was freeze-dried under reduced pressure to provide a brown residue in about 25% yield.

Urinary Excretion Study——Following an intraperitoneal administration of Salviae miltiorrhizae Radix extract, urine collections were made every 3 h to determine urinary excretions of urea, creatinine, and electrolytes. Urea was determined by the method of Archibald.[8] Creatinine was determined by the Folin–Wu method,[9] with a commercial reagent (Creatinine-Test Wako) supplied by Wako Pure Chemical Industries, Ltd., Osaka, Japan. Sodium and potassium were measured with an electrolyte measurement apparatus (AHS/Japan Corporation, Tokyo, Japan) based on the ion electrode method. Inorganic phosphate and calcium were determined by using the molybdenum blue method[10] and *ortho*-cresol-phthalein complex compound method[11] with commercial reagents (Phosphor B-Test Wako and Calcium C-Test Wako, respectively) purchased from Wako Pure Chemical Industries.

Renal Function Study——GFR, RPF, hematocrit value (Ht), and RBF values were obtained at 5.5—6 h after intraperitoneal administration of Salviae miltiorrhizae Radix extract. GFR and RPF were measured by means of a renal clearance test using a single intravenous administration of sodium thiosulfate or sodium *para*-aminohippurate, respectively, as an indicator.[12,13] At 25 min after intravenous administration of sodium thiosulfate or sodium *para*-aminohippurate, the bladder was reflexly emptied by having each rat inhale ether for 3—5 s. The urine thus voided was discarded. After a further 30 min, the urine was collected, by reflex bladder emptying induced by ether inhalation. Blood samples were taken from conscious rats by heart puncture at the middle of the clearance test period. Thiosulfate and *para*-aminohippurate were determined by titrimetry and colorimetry, respectively. RBF was calculated on the basis of RPF and Ht by using the equation shown below. Ht was determined with a hematocrit measurement apparatus, model KH-120A (Kubota Co., Ltd., Tokyo, Japan).

$$\mathrm{RBF}=\frac{\mathrm{RPF}}{1-\mathrm{Ht}}\ (\mathrm{ml/min})$$

Statistics——The significance of differences between the control and Salviae miltiorrhizae Radix extract-treated groups was tested by the use of Student's *t*-test. A p value greater than 0.05 was considered not to be significant.

Results

Urine Volume

As shown in Table I, urine volume increased by about 3.9-fold and 2.0-fold in comparison with the controls at 3—6 h and 6—9 h after treatment with Salviae miltiorrhizae Radix extract, while at 0—3 h and 9—12 h significant changes were not observed. The total urine volume throughout the experiment was higher in the extract-treated rats than in control rats (6.52 ml/12 h *vs.* 3.95 ml/12 h; 65% increase).

TABLE I. Effect of Salviae miltiorrhizae Radix Extract on Urine Volume

Time (h)	Material	Urine volume (ml/3 h)
0—3	Control	1.35±0.14
	Extract	1.16±0.29
3—6	Control	0.76±0.19
	Extract	2.93±0.45[b]
6—9	Control	0.84±0.10
	Extract	1.72±0.35[a]
9—12	Control	1.00±0.15
	Extract	0.71±0.15

Values are means±S.E. of 6 rats. *a*) Significantly different from the control value, $p<0.05$, *b*) $p<0.01$.

TABLE II. Effect of Salviae miltiorrhizae Radix Extract on Urinary Urea and Creatinine Excretions

Time (h)	Material	Urea (mg/3 h)	Creatinine (mg/3 h)
0—3	Control	99.1±9.6	0.93±0.12
	Extract	113.7±13.8	0.98±0.16
3—6	Control	89.0±10.8	0.70±0.13
	Extract	141.2±20.7[a]	1.13±0.08[a]
6—9	Control	78.7±11.6	0.66±0.06
	Extract	112.8±23.7	0.90±0.18
9—12	Control	50.4±5.1	0.64±0.07
	Extract	54.0±6.5	0.59±0.03

Values are means±S.E. of 6 rats. *a*) Significantly different from the control value, $p<0.05$.

Excretion of Urea and Creatinine

Treatment with Salviae miltiorrhizae Radix extract significantly increased urinary urea excretion by 59% from 89.0 mg/3 h to 141.2 mg/3 h at 3—6 h and tended to increase it at 0—3 h and 6—9 h, as shown in Table II. The total urinary urea amount in the extract-treated group throughout the experimental period also increased by about 33% as compared with the control group (421.7 mg/12 h *vs.* 317.2 mg/12 h). Similarly, Salviae miltiorrhizae Radix extract significantly increased urinary creatinine excretion by 61% from 0.70 mg/3 h to 1.13 mg/3 h at 3—6 h, and tended to increase urinary creatinine excretion at 6—9 h (Table II). The total urinary creatinine throughout the experiment was higher in the extract-treated rats than in control rats (3.60 mg/12 h *vs.* 2.93 mg/12 h; 23% increase).

Excretion of Electrolytes

As shown in Table III, the administration of Salviae miltiorrhizae Radix extract resulted in remarkable increases of urinary sodium excretion by 494%, 484%, and 255% at 3—6 h, 6—

TABLE III. Effect of Salviae miltiorrhizae Radix Extract on Urinary Electrolyte Excretion

Time (h)	Material	Na (μM/3 h)	K (μM/3 h)	P (μg/3 h)	Ca (μg/3 h)
0—3	Control	126.8±21.2	223.8±30.1	67.5±14.8	84.1±14.9
	Extract	115.3±23.7	214.5±42.6	38.9±17.7	94.2±13.6
3—6	Control	22.8±6.7	130.4±32.5	159.1±34.0	39.8±9.4
	Extract	135.4±17.9[c)]	436.9±64.2[b)]	330.0±53.5[a)]	96.1±8.8[b)]
6—9	Control	11.3±2.5	120.6±10.2	201.4±18.8	39.3±6.4
	Extract	66.0±20.8[a)]	234.4±50.4[a)]	331.4±46.4[a)]	70.7±18.6
9—12	Control	8.6±1.8	140.8±17.9	261.0±31.0	47.4±5.1
	Extract	30.5±8.9[a)]	95.6±27.3	274.9±26.6	32.9±4.6[a)]

Values are means±S.E. of 6 rats. *a*) Significantly different from the control value, $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

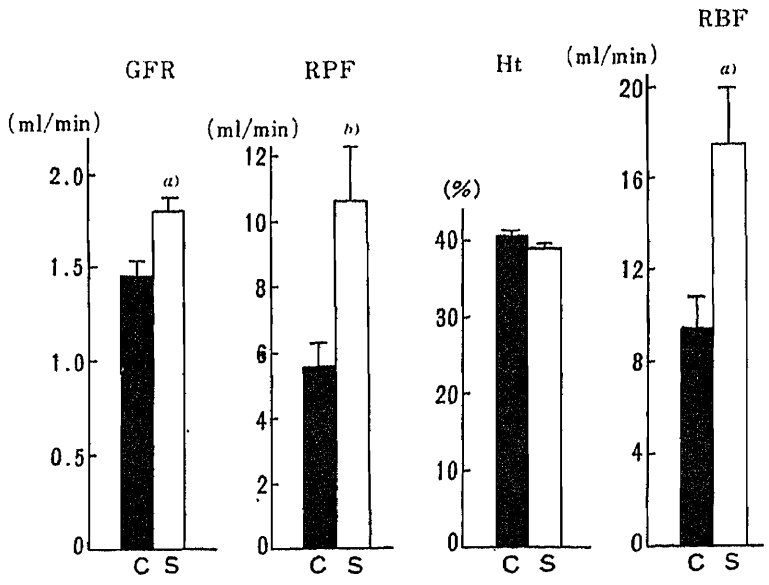

Fig. 1. Effect of Salviae miltiorrhizae Radix Extract on Renal Function Tests

C, control group; S. Salviae miltiorrhizae Radix extract-treated group. GFR, glomerular filtration rate; RPF, renal plasma flow; Ht, hematocrit value; RBF, renal blood flow. Values are means±S.E. of 6 rats. *a*) Significantly different from the control value, $p<0.05$, *b*) $p<0.01$.

9 h, and 9—12 h, respectively. The total urinary sodium excretion throughout the experimental period in the extract-treated group was significantly increased by about 105% as compared with the control group. Similarly, the extract significantly increased the urinary excretion of potassium by 235% and 94% at 3—6 h and 6—9 h, respectively. However, at 9—12 h, potassium excretion was decreased, though not significantly (Table III). The total urinary potassium excretion in the extract-treated group was higher than that of control rats (981.4 μM/12 h *vs.* 615.6 μM/12 h; 59% increase). The administration of the extract significantly increased urinary inorganic phosphate excretion at 3—9 h as compared with the control, as shown in Table III. However, at 9—12 h, the increase in inorganic phosphate excretion was not significant compared with the control. The extract increased the urinary excretion of inorganic phosphate throughout the experimental period by 42% from 689.0 μg/12 h to 975.2 μg/12 h. Urinary calcium excretion remained above the control at 0—9 h, but the difference was significant only at 3—6 h. However, at 9—12 h, the urinary calcium excretion was significantly decreased (Table III). The total urinary calcium in the extract-treated group was increased by about 40% over 0—12 h, compared with the control group.

GFR, RPF, and RBF

Treatment with Salviae miltiorrhizae Radix extract significantly increased GFR by 24% from 1.47 ml/min to 1.82 ml/min, as shown in Fig. 1. In addition, the extract increased RPF by 89% from 5.60 ml/min to 10.60 ml/min ($p<0.01$). On the other hand, there was no statistically significant difference between the control and extract-treated groups with regard to Ht. When RBF was calculated on the basis of RPF and Ht, RBF was 9.50 ml/min in the control rats and 17.45 ml/min in the extract-treated rats; this difference is significant. The extract significantly increased RBF by 84% ($p<0.05$).

Discussion

The acute administration of Salviae miltiorrhizae Radix extract markedly increased urine volume, and urea, creatinine, sodium, potassium, and inorganic phosphate excretions. The observed diuresis, natriuresis, kaliuresis, and phosphaturia were striking, with values increased 3.9-, 5.9-, 3.4-, and 2.1-fold at 3—6 h in the extract-treated rats as compared with the control. The natriuresis was not accompanied with potassium retention, so that a marked increase in potassium excretion was observed. This implies that the natriuretic effect of Salviae miltiorrhizae Radix extract is not mediated *via* reduced aldosterone secretion, a conclusion which is supported by the results that no changes in the renin–angiotensin system and aldosterone level were observed (data not shown). Phosphaturia was also noted. Since phosphate is primarily reabsorbed in the proximal tubule,[14] this suggests that the extract may interfere with proximal tubular phosphate transport.

On the other hand, the acute administration of Salviae miltiorrhizae Radix extract caused significant increases in GFR, RPF, and RBF. These results indicate that the extract appreciably affects renal function. The increases in urinary urea and creatinine induced by the extract may be attributed to the increases in GFR, RPF, and RBF. The increased RBF in the acute experiment is consistent with the results in the chronic experiment reported previously.[7]

GFR is influenced by various factors.[15] In particular, changes in the mean transcapillary hydrostatic pressure difference and glomerular ultrafiltration coefficient might explain the increase in GFR. The mean transcapillary hydrostatic pressure difference is thought to be altered by changes in either afferent or efferent arteriolar tone.[16] In our experiments, GFR increased with a concomitant increase in RBF, thus indicating that the degree of afferent arteriolar dilation exceeds that of efferent arterioles. The increase in GFR by Salviae miltiorrhizae Radix extract may have produced a decrease of fractional reabsorption of

sodium in the renal tubules and induced an increase in the delivery of sodium. There is also the possibility that the intrarenal hemodynamic alteration may affect the regulation of sodium balance within the kidney. These effects of the extract are similar to the natriuretic action of aminophylline, which is due in part to a tubular blockade of sodium reabsorption and in part to an enhancement of the hemodynamics.[17]

Though the urinary excretion and renal function were improved in normal rats by Salviae miltiorrhizae Radix extract, it remains to be seen whether the extract is effective in experimentally uremic rats and whether similar effects on the renal elimination of uremic toxins will be observed during chronic administration.

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Opsonin-Independent Phagocytosis of Periodate-Treated Sheep Red Blood Cells by Macrophages

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The opsonin-independent recognition of periodate-treated sheep red blood cells (P-SRBC) by macrophages was studied by observation of phagocytosis and the mechanism of the recognition was compared with those for other particles. Thioglycollate-elicited guinea pig peritoneal macrophages time-dependently ingested sheep red blood cells (SRBC) treated with periodate, as well as immunoglobulin G-coated sheep red blood cells (IgG-SRBC), zymosan (Z), serum-treated zymosan (STZ) and latex beads. Trypsinization of macrophages decreased the ingestion of P-SRBC to under 50% of the value of untreated cells and virtually abolished the ingestion of Z and STZ at a concentration that did not inhibit the phagocytosis of IgG-SRBC and latex beads. The decreased activities for P-SRBC recovered to 80% of the control value on incubation of the macrophages for 5h at 37°C. The restoration of the ability to ingest P-SRBC following trypsin digestion was inhibited by cycloheximide and tunicamycin. Pretreatment of macrophages with ConA dose-dependently decreased ingestion of P-SRBC to under 50% of the original level, but did not decrease the internalization of IgG-SRBC and STZ. Rabbit anti-guinea pig peritoneal macrophage IgG abolished the ingestion of Z and caused marked and slight decreases of phagocytosis of IgG-SRBC and P-SRBC, respectively.

These results indicated that the site of recognition of P-SRBC on the macrophage cell membrane could be composed of glycoproteins and is distinct from the receptors for C3b and Z. The role of plasma membrane components on macrophages in the action of opsonin-independent recognition is discussed in relation to the opsonin-mediated recognitions.

Keywords——macrophage; phagocytosis; opsonin-independent recognition; periodate-treated red blood cell; trypsin digestion; anti-macrophage IgG; zymosan; concanavalin A

Introduction

It is known that macrophages possess membrane receptors for Fc portion of immunoglobulin G (IgG) and C3 component of the complement system, which enable the cells to recognize and ingest foreign particles.[1] Without these receptors, phagocytic recognition can still take place by direct binding of foreign substances such as bacteria, latex beads, zymosan (Z) and chemically altered red blood cells to the membrane of phagocytic cells.[2] It is important to know the chemical principles of opsonin-free recognition by phagocytes in order to understand the self-defense mechanisms against invasion by foreign substances, especially before initiation of the immunological responses. Previously, Rabinovitch[3] reported that red blood cells modified by exposure to periodate exhibit typical rosette formation at the macrophage cell surface and recently, Capo *et al.*[4] described the opsonin-independent phagocytosis of aldehyde-treated erythrocytes by macrophages, though the chemical properties of the binding site were not elucidated clearly.

The present studies were performed to clarify the mechanisms which govern the discriminatory qualities of macrophages in opsonin-independent recognition. For this

purpose, we have examined the effect of periodate treatment of sheep red blood cells (SRBC) on the phagocytosis by and binding to guinea pig peritoneal macrophages, and the properties of the recognition site for periodate-treated SRBC (P-SRBC) were studied and compared with those of the sites for other particles such as IgG-coated SRBC (IgG-SRBC), Z, serum-treated zymosan (STZ) and latex beads. It was indicated that P-SRBC could be ingested by guinea pig macrophages, as could the other particles tested, and the site for recognition of P-SRBC was distinct from the sites for other particles in terms of sensitivity to trypsin digestion, and responses to treatment with concanavalin A (ConA) and sensitization with anti-macrophage rabbit IgG antibodies. The chemical properties of the recognition site for P-SRBC are discussed in relation to those of the receptors for IgG, C3b and Z.

Materials and Methods

Macrophages——Thioglycollate-elicited peritoneal macrophages were obtained as described by Griffin *et al.*[5] Male or female Hartley guinea pigs weighing about 300 g were injected intraperitoneally with 20 ml of Brewer's thioglycollate medium and 4 d later, peritoneal exudate cells (PEC) were harvested with Dulbecco's phosphate buffer. After disruption of contaminating erythrocytes with hypotonic buffer, cells were washed with Dulbecco's phosphate buffer and suspended in Eagle's minimum essential medium (MEM) to a concentration of 1×10^7 PEC/ml. Then 50 μl of the PEC cell suspension was placed in 10 mm flat-bottomed Linbro tissue culture wells in a final volume of 0.5 ml and the plate was incubated for 2 h at 37 °C in a humid chamber under an atmosphere of 5% CO_2 to permit adherence of macrophages. After elimination of non-adherent cells, 0.5 ml of MEM was added and phagocytosis and rosette formation were examined. Over 95% of cells remained viable during the experiment as assessed by exclusion of 0.5% trypan blue.

Assay of Phagocytosis——Macrophage monolayers were incubated with 5×10^6 IgG-SRBC, P-SRBC, latex beads or Z or the same number of Z treated with guinea pig serum (STZ) in 0.5 ml of MEM, usually for 60 min at 37 °C in a humid chamber under an atmosphere of 5% CO_2. After being rinsed four times with warmed MEM to remove free particles, the cells that had ingested particles were counted under a microscope. Phagocytic activities were determined by scoring macrophages incorporating one or more particles. At least 100 macrophages were examined, and the results were expressed as the percentage of particle-carrying cells.

Assay of Attachment——Suspensions of target particles, 0.05 ml were added to macrophage monolayers as mentioned above and incubated for 15 min at 37 °C. After being washed with MEM, monolayers were examined for binding of particles under a microscope. Binding activities were calculated by scoring cells carrying one or more particles. At least 100 macrophages were examined.

IgG-SRBC——A 0.5% SRBC suspension in phosphate-buffered saline (PBS) 2 ml was sensitized with 0.22 mg (a subagglutinating dose) of rabbit IgG antibodies for 20 min at 37 °C and after being washed with PBS, the resulting IgG-SRBC were resuspended in MEM at 1×10^8 cells/ml.

P-SRBC——SRBC were usually treated with 4 mM sodium metaperiodate in saline containing 10 mM acetate buffer, pH 5.5 for 20 min at 37 °C in the dark. After thorough washing with saline, the P-SRBC were suspended in PBS at 10^8/ml and stored at 4 °C. They were used within a week.

Z and STZ——Z suspended in PBS at 10 mg dry weight/ml was homogenized in a Tefron homogenizer, incubated for 60 min in boiling water, suspended in saline and stored at −80 °C. For each experiment, the particles were washed with PBS three times by centrifugation and suspended at 5 mg/ml in MEM. STZ was prepared as follows; one ml of Z suspension (5 mg/ml saline) was incubated with 0.2 ml of fresh guinea pig serum in gelatin veronal-buffered saline for 30 min at 37 °C. After being washed with PBS three times, STZ thus obtained was suspended in 1 ml of MEM.

Anti-SRBC Rabbit IgG Antibodies——Rabbits were immunized intravenously five times with 5 ml of 10% SRBC at intervals of 3 d and were bled 5 d after the last injection. The IgG fraction of antiserum was prepared by precipitation with 50% saturation of ammonium sulfate followed by chromatography on a diethylaminoethyl (DEAE)-cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.4. The unadsorbed fraction was collected and used as anti-SRBC IgG antibodies for sensitization.

Rabbit IgG-Antibodies against Guinea Pig Peritoneal Macrophages——Guinea pig peritoneal exudate cells were cultured for 2 h on a 100 mm tissue culture dish (Falcon no. 3003) and after removal of non-adherent cells the adherent cells were harvested with a rubber policeman and suspended in PBS. The cell suspensions were mixed with an equal volume of complete Freund's adjuvant and used as an immunogen. A rabbit was immunized with four weekly injections of 3.75 mg each of the macrophage protein. The serum was obtained 10 d after the last injection and the IgG fraction of the antiserum was isolated by precipitation with ammonium sulfate followed by chromatography on a DEAE-cellulose column. The IgG antibodies obtained were absorbed with SRBC three times to remove antibodies against SRBC.

Determination of Protein——Proteins were determined according to the method of Lowry *et al.* using bovine serum albumin as a standard.[6]

Reagents——Cycloheximide, a product of Tanabe Pharmaceutical Industry, was dissolved in saline at 1 mg/ml and kept for up to a week at 4 °C. Tunicamycin and ConA were obtained from Sigma Chemicals and dissolved in 5 mM NaOH to 1 mg/ml and in saline to 10 μg/ml, respectively. The solutions were kept for up to a week at 4 °C. Phospholipase c was purchased from Sigma Chemicals and dissolved in saline at 10 units/ml for each experiment. Latex breads, 3.5 μm in diameter, were purchased from Dow, Indianapolis, U.S.A., and after being washed with PBS three times, the particles were suspended in PBS at 10^8/ml.

Results

Phagocytosis of P-SRBC

Rabinovitch reported that red blood cells modified by exposure to periodate were bound to the macrophage cell surface in the absence of opsonin.[3] First, we examined the effect of periodate treatment of SRBC on the phagocytosis by macrophages under various conditions. SRBC were treated with various concentrations of sodium metaperiodate at 37 °C in saline containing 10 mM acetate buffer, pH 5.5, for 20 min and after being washed with saline, the P-SRBC obtained were added to macrophage monolayers in a ratio of 10 : 1. After 60 min of incubation at 37 °C in MEM, monolayers were washed thoroughly to remove free particles, and phagocytosis was examined under a microscope. As can be seen in Fig. 1, guinea pig peritoneal macrophages ingested P-SRBC; over 90% of the cells ingested one or more particles when SRBC were treated with 10 mM periodate. The effect of the ratio of the number of target particles to that of phagocytes was estimated and it was found that at the ratio of 10 : 1, about 90% of macrophages ingest one or more P-SRBC.

Then, the time course of ingestion of P-SRBC was followed in comparison with that mediated by Fc receptors and that by C3b, assessed in terms of ingestion of STZ as described previously.[7] SRBC treated with 5 mM periodate were ingested avidly by macrophages, as were

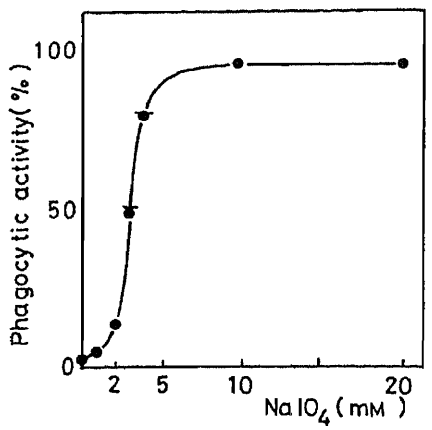

Fig. 1. Ingestion of Periodate-Treated Sheep Red Blood Cells (P-SRBC) by Macrophages

SRBC (4×10^9) were treated with various concentrations of periodate in 4 ml of 20 mM acetate-buffered saline, pH 5.5 for 20 min at 37 °C in the dark. After thorough washing with PBS, the P-SRBC were suspended in MEM at 10^8 cell/ml. P-SRBC (5×10^6) cells were added to macrophage monolayers and incubated for 60 min in a CO_2 incubator as described in Materials and Methods. After washing away of free particles, phagocytosis was estimated under a microscope. Results are expressed as percentage of cells carrying one or more particles. Values are mean ± S.D. of triplicate determinants.

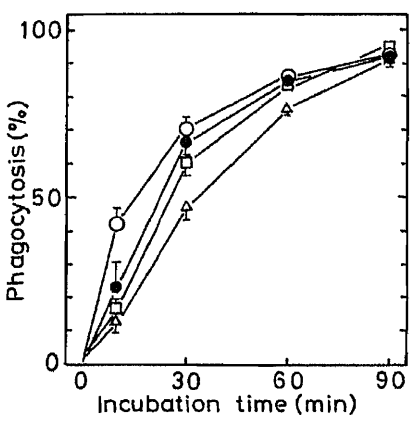

Fig. 2. Phagocytic Activities of Macrophages towards Various Particles

Macrophage monolayers were incubated with 5×10^6 IgG-SRBC, P-SRBC, latex beads, or Z in 0.5 ml of MEM for various intervals at 37 °C in a 5% CO_2 atmosphere. After elimination of free particles, phagocytic activities were estimated. Results are expressed as described in the legend to Fig. 1. Values are mean ± S.D. of three to five determinations. IgG-SRBC ($n=3$), ○; P-SRBC ($n=5$), □; Z ($n=4$), △; latex beads ($n=3$), ●.

the other particles, IgG-SRBC, Z, STZ and latex beads (Fig. 2).

Effect of Trypsin Digestion on the Phagocytic Activities

To identify the chemical principle of opsonin-independent recognition of P-SRBC, macrophage monolayers were treated with trypsin at 37 °C, then washed with PBS, and the phagocytic activities were estimated. When the monolayers were treated with 1 mg/ml of trypsin, phagocytosis of P-SRBC decreased time-dependently to under 50% of the original value, while ingestion of IgG-SRBC was not affected significantly until 90 min after the start of the treatment (data not shown). Next, macrophage monolayers were treated with various concentrations of trypsin for 60 min and washed with PBS, and the phagocytic activities for P-SRBC, IgG-SRBC, STZ and Z were estimated. As can be seen in Fig. 3, ingestion of P-SRBC decreased dose-dependently on trypsinization of the cells, and it was found that the binding sites for Z and STZ were more sensitive to trypsin digestion, as the activities towards these particles were abolished by treatment with 0.1 and 0.5 mg/ml of trypsin, respectively. On the other hand, ingestions of IgG-SRBC and latex beads were not affected significantly even after treatment with up to 2.0 mg/ml of trypsin. These results suggested that the recognition site for P-SRBC is distinct from those for other particles. The phenomenon of phagocytsis can be divided into two phases, attachment and subsequent internalization. Thus, to determine whether trypsinization of macrophage monolayers affects the adherence of P-SRBC or the subsequent internalization, adherence of the particles was examined. As can be seen in Fig. 4, after trypsinization, attachment of P-SRBC to macrophages decreased to under 50% of the original value. Ögmundsdottir *et al.* reported that *C. parvum* binds to mouse peritoneal exudate cells in the absence of opsonin, and trypsin digestion of the phagocytes caused a decreased binding of the bacteria, while the activity was restored to the normal value after

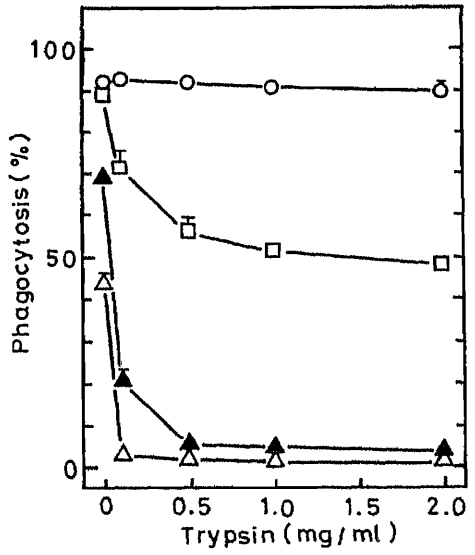

Fig. 3. Dose-Effect Relationship of Trypsin Action on the Ability of Macrophages to Ingest P-SRBC, IgG-SRBC, Z and STZ

Macrophage monolayers were treated with various concentrations of trypsin for 60 min and after being washed with MEM, the monolayers were incubated with IgG-SRBC, P-SRBC, Z or STZ for 60 min. After elimination of free particles, phagocytosis was examined and evaluated as described in the legend to Fig. 1. Values are mean ± S.D. IgG-SRBC ($n=3$), ○; P-SRBC ($n=10$), □; Z ($n=3$—10), △; STZ ($n=3$), ▲.

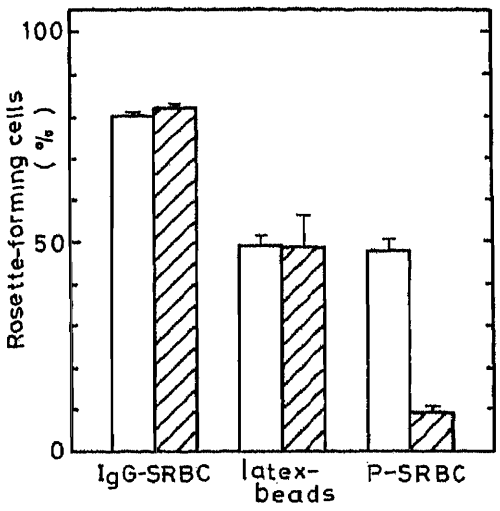

Fig. 4. Effect of Trypsin Digestion on Rosette Formation

Macrophage monolayers were treated with 1.0 mg/ml of trypsin in MEM for 60 min at 37 °C then washed with MEM. IgG-SRBC, latex beads, or P-SRBC were added and incubated for 15 min at 37 °C. After elimination of nonadherent particles, rosette-forming cells were counted under a microscope. The results were expressed as percentage of macrophage carrying one or more particles. Values are mean ± S.D. of triplicate determinants. Open and shadowed columns show the results for control and trypsin-treated cells, respectively.

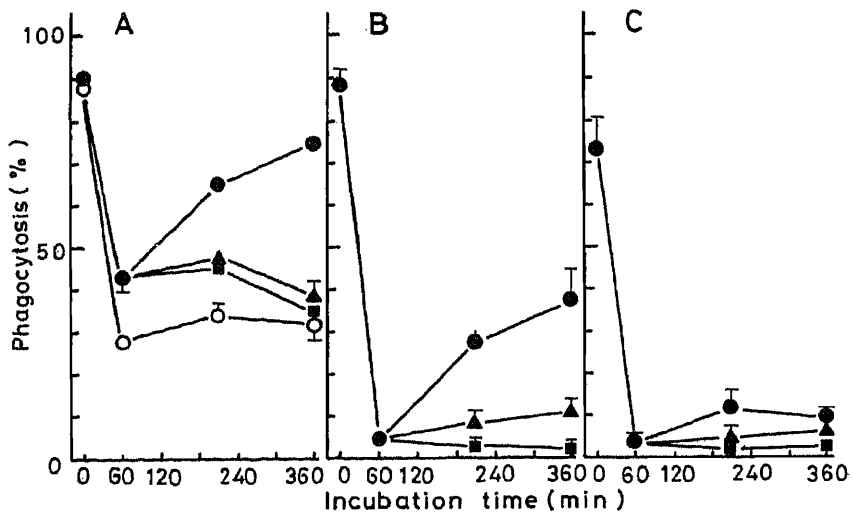

Fig. 5. Recovery of the Phagocytic Activities for P-SRBC, STZ and Z after Trypsinization

Macrophage monolayers were treated with 1.0 mg/ml of trypsin in MEM for 60 min at 37 °C then washed with MEM, and incubated for a further 120 or 300 min in MEM in the absence (●) or presence of tunicamycin, 1.0 μg/ml (▲) or cycloheximide, 100 μg/ml (■), at 37 °C. The monolayers were washed with MEM and phagocytic activities for P-SRBC (A), STZ (B) and Z (C) were estimated as described in the legend to Fig. 1. Open circles showed the activities of macrophages pretreated with 10 μg/ml of phospholipase c for 30 min at 37 °C in MEM followed by trypsinization. Values are mean ± S.D. for triplicate determinations.

incubation at 37 °C following trypsin digestion.[8] Thus, to elucidate the chemical properties of the binding site for P-SRBC, trypsinized macrophage monolayers were washed with PBS and incubated at 37 °C in MEM, and phagocytic activity was followed. As shown in Fig. 5A, the activity of trypsinized macrophages for P-SRBC was restored to 80% of the original value by incubation for 5 h and the restoration of the activity was inhibited by the addition of cycloheximide or tunicamycin. When macrophage monolayers were pretreated with phospholipase c and then treated with trypsin, ingestion of P-SRBC decreased to under 30% of the original value and no restoration was observed during 5 h of incubation. These results suggested that the recognition site for P-SRBC on macrophage cell membrane is composed of proteins, glycosides and phospholipids. Phagocytic activities of trypsinized macrophages for STZ recovered similarly to about 50% of the original value after 5 h of incubation and the recovery was also inhibited by cycloheximide and tunicamycin (Fig. 5B). On the other hand, after trypsinization, the phagocytic activity for Z increased only slightly, and the recovery was inhibited by cycloheximide and tunicamycin (Fig. 5C). Untreated macrophage maintained constant values of phagocytic activities for these targets during 6 h of incubation, even in the presence of cycloheximide or tunicamycin (data not shown).

Effects of ConA

As mentioned above, the macrophage binding site for P-SRBC could be composed of glycoproteins. To examine whether glycoproteins participate in the recognition of P-SRBC, the effect of ConA on the phagocytosis was examined. The macrophage monolayers were treated with 10 μg/ml of ConA for various intervals then washed with PBS, and the phagocytic activities were determined. Treatment of macrophages with ConA decreased the ingestion of P-SRBC time dependently whereas phagocytosis of IgG-SRBC was not changed until 90 min after the start of treatment (data not shown). Next, macrophage monolayers were treated with various concentrations of ConA in MEM for 30 min at 37 °C and then washed with PBS, and the phagocytic activities for various particles were determined. Ingestion of P-

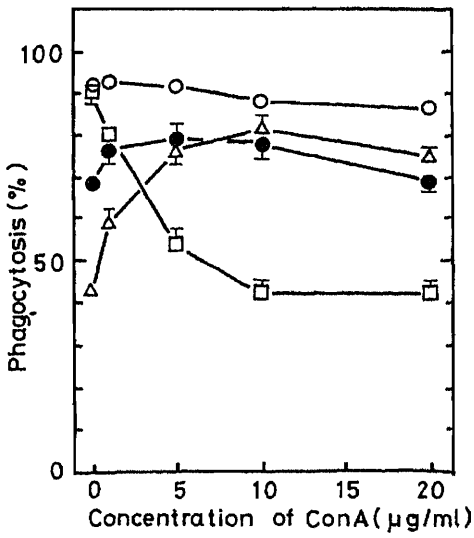

Fig. 6. Dose–Response Relationship in the Effect of ConA Pretreatment on the Phagocytic Activities

Macrophage monolayers were treated with various concentrations of ConA in MEM for 30 min at 37 °C, then washed with MEM, and incubated with IgG-SRBC (○), P-SRBC (□), Z (△) and STZ (●) as described in the legend to Fig. 1. Each value is the mean±S.D. of triplicate experiments.

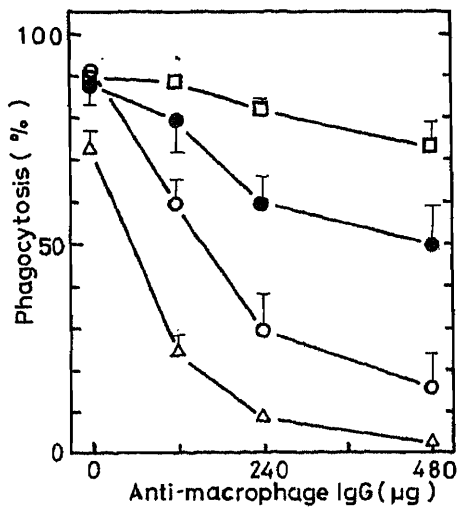

Fig. 7. Effect of Anti-macrophage IgG-Antibodies on the Phagocytic Activities

Macrophage monolayers were incubated with 0, 120, 240, 360 and 480 μg of anti-macrophage rabbit IgG in 0.5 ml of MEM for 30 min at 37 °C. Then washed with MEM. Phagocytic activities for various particles were estimated as described in the legend to Fig. 1. Values are mean±S.D. IgG-SRBC ($n=3$), ○; P-SRBC ($n=4$), □; Z ($n=3$), △; STZ ($n=4$), ●.

SRBC was decreased markedly by pretreatment of macrophages with increasing amounts of ConA, while Fc- and C3-mediated phagocytosis were not affected significantly (Fig. 6). In contrast, increased ingestion of Z was observed in the region of low concentration of ConA.

Effect of Anti-macrophage IgG-Antibodies

To examine whether the site for recognition of P-SRBC could be discriminated immunologically from the sites for other particles, macrophage monolayers were treated with rabbit IgG-antibodies against guinea pig peritoneal macrophages and the phagocytic activity for P-SRBC was determined and compared with the activities for various other particles. As can be seen in Fig. 7, after sensitization with the antibodies, the phagocytic activity for Z decreased dependently on the amount of antibodies and was abolished at the highest concentration of antibodies tested. The activities for IgG-SRBC and STZ decreased to under 20% and 60% of the original values, respectively, with the same amount of antibodies. In contrast, phagocytosis of P-SRBC decreased only slightly even at the highest concentration of IgG used in this experiment.

Discussion

The opsonin-independent recognition and ingestion of particles by phagocytes are essential for elimination of senescent self-materials and foreign substances, especially before the induction of immune responses. Rabinovitch has already described the attachment of P-SRBC to macrophages, but the experimental conditions for treatment of SRBC with periodate and the properties of the binding site on phagocytes were not studied in detail.[3] In this paper, we have demonstrated that when SRBC were treated with periodate in acetate buffer, the resultant P-SRBC could be ingested by macrophages in proportion to the

concentration of periodate used for modification. It was found that the binding sites on macrophage cells were sensitive to trypsin digestion and that after elimination of the trypsin, phagocytic activity was restored to 80% of the original value by incubation for 5 h at 37 °C. Furthermore, the restoration of the activity was inhibited by cycloheximide and tunicamycin. These results imply that the binding sites for P-SRBC on macrophage cells involve glycoproteins. This conclusion was supported by the observation that ingestion of P-SRBC was inhibited by pretreatment of macrophages with ConA. Pretreatment of macrophages with phospholipase c before trypsinization decreased the activities of the cells markedly, but the decrease was not observed after treatment with the enzyme alone. It is possible that phospholipid is essential for assembly of the binding site on macrophage cell surface.

The binding site for P-SRBC is distinct from the sites for other particles in several respects. Namely, IgG receptors are insensitive to trypsin digestion and ingestion of latex beads is not affected by trypsin digestion or anti-macrophage IgG. On the other hand, ingestion of Z was decreased markedly by treatment with a low concentration of trypsin and the activity was not restored significantly by incubation at 37 °C. In contrast, C3b receptors were inactivated by trypsin digestion and restored by subsequent incubation at 37 °C as binding sites for P-SRBC, but were distinct from the sites for P-SRBC in that preincubation of macrophages with increasing concentrations of ConA decreased the ingestion of P-SRBC markedly while ingestion of STZ was not affected significantly by the same treatment. Concerning the effect of pretreatment of macrophages with phytohemagglutinins, wheat germ agglutinin (WGA) and ricinus commnis agglutinin-1 (RCA-1) decreased the ingestion of P-SRBC as did ConA, while peanut agglutinin (PNA), soybean agglutinin (SBA), maclura pomifera ggglutinin (MPA), dolichos biflorus agglutinin (DBA) and bandeiraea simplicifolia agglutinin-1 (BSA-1) has no effect on the ingestion of P-SRBC (data not shown). These results further support the conclusion that glycoproteins participate in the ingestion of P-SRBC by macrophages. After treatment of macrophages with these lectins, attachment to or ingestion by macrophages of native SRBC was not observed.

Concerning opsonin-independent phagocytosis, Capo *et al.* reported that treatment of red blood cells with aldehydes allowed the cells to be ingested by rat macrophages, and they suggested that aldehyde-mediated phagocytosis of SRBC was due to both increased local rigidity and modified hydrophobicity of the cell surface induced by cross-linking of the cell membrane structures.[4] In 1978, Czop *et al.* reported that human monocytes ingest Z and heterologous red blood cells without exogenous opsonin and demonstrated that these recognition sites were sensitive to trypsin digestion.[9] In the present work the binding site for P-SRBC was discriminated clearly from the site for Z as follows. First, after treatment of macrophages with ConA, phagocytosis of P-SRBC but not of Z decreased dose-dependently; second, sensitization of macrophages with antibodies caused marked and slight decreases of the ingestion of Z and P-SRBC, respectively; third, the site for Z was destroyed by treatment with a low concentration of trypsin, in contrast to the site for P-SRBC, and the activity was not restored during 5 h of incubation. Treatment of macrophages with increasing concentrations of trypsin reduced the phagocytosis of P-SRBC to one-half of the original value while the activity for Z was lost entirely (Fig. 3). It is possible that macrophages possess two kinds of binding site for P-SRBC, and one of which is insensitive to trypsin digestion. Treatment of SRBC with periodate could induce chemical modification of glycoside chains, lipids and proteins on the cell membranes, resulting in recognition by macrophages, though the nature of the modifications is not known chemically. The relationship of phagocytosis to the stimulation of biological responses such as superoxide anion release and lysosomal enzyme release needs to be examined. The mechanisms of the opsonin-independent recognition of particles by macrophages, especially the protein chemical properties of the binding sites for P-SRBC and Z on the phagocytes, are under investigation.

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Singlet Oxygen-Producing Activity and Photodynamic Biological Effects of Acridine Compounds

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The singlet oxygen-producing activities and photodynamic biological activities of acridine compounds were compared. Singlet oxygen was trapped by 2,2,6,6-tetramethyl-4-piperidone (TEMP), and 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPO) produced was detected by electron spin resonance (ESR) spectrometry. TEMPO production was inhibited by NaN_3 and enhanced in D_2O, confirming it to be an adequate ESR spectroscopic indicator for singlet oxygen.

Comparison of TEMPO-producing activities revealed that acriflavine and proflavine, which are the most potent photosensitizers both in cell inactivation and petite induction of yeast, produced the most intense and long-lived ESR signals. However there was no clear difference of TEMPO-producing activity between biologically ineffective acridines such as acridine and quinacrine and effective ones such as acridine yellow and 6,9-diamino-2-ethoxyacridine.

These results suggested that the differences observed in the photodynamic biological effects among the acridine compounds depend mainly on the differences of drug distribution and only partly on the differences of singlet oxygen-producing activity of the dye molecule itself.

Keywords——singlet oxygen production; photodynamic action; acridine compound; ESR detection; 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPO); yeast; *Saccharomyces cerevisiae*; cell inactivation; petite induction

A variety of compounds have been demonstrated to be active photo-sensitizers in different organisms.[1] In previous studies,[1a,2] acriflavine (AF) was identified as an active photo-sensitizer in yeast. Irradiation of AF-pretreated cells with common fluorescent lamps brought about cell inactivation, petite induction and an increase in the reversion rate from tryptophan auxotrophy to prototrophy.[2b] These biological effects were suggested to be induced by the type II photodynamic action mediated by singlet oxygen.[2] Singlet oxygen production has been detected indirectly in terms of nitroxide radical production by the electron spin resonance (ESR) method.[3] Using this method, the production of the nitroxide radical in photo-irradiated AF solution was demonstrated.[4] Comparison of the photodynamic activities of acridine compounds[5] revealed that proflavine (PF), acridine yellow (AY), 3,6-diamino-2-ethoxyacridine (DAEA) and acridine orange (AO), in addition to AF, were highly toxic and mutagenic, but acridine (A), quinacrine (Q) and 9-aminoacridine (AA) were not. These findings led us to compare the singlet oxygen-producing activities and the photodynamic biological activities among acridine compounds.

The results presented here indicate that nitroxide radical production in photo-irradiated dye solution is closely related to singlet oxygen production. All acridines used here could produce nitroxide radical by photodynamic action. AF or PF, which induce intense and various photodynamic biological effects, produced the most intense and long-lived ESR signals of nitroxide radical.

Materials and Methods

Detection of Singlet Oxygen——Details of the method were given in the previous report.[4]

Preparation of Reaction Mixture——All the solution were prepared in glass using redistilled water. The components of the standard reaction mixture were 2×10^{-2} M 2,2,6,6-tetramethyl-4-piperidone (TEMP), 10^{-5} M dye solution and 1/15 M phosphate buffer (pH 9.2). In order to determine the inhibitory effect of sodium azide, 50 mM NaN_3 was added. An anoxic sample was prepared as follows. A reaction mixture containing 2×10^{-2} M TEMP, 10^{-5} M AF and 1/15 M phosphate buffer (pH 9.2) was frozen by the use of liquid nitrogen in a quartz tube with a capillary side branch. Then the tube was evacuated, thawed and frozen repeatedly in order to remove molecular oxygen thoroughly without changing the volume of the contents. Finally the neck of the evacuated tube was heat-sealed. A part of the contents (about 50 μl) was decanted into the capillary branch and the neck of the capillary was heat-sealed. A D_2O sample was prepared as follows. First, 0.5 ml of 2/15 M phosphate buffer (pH 9.2) and a mixture of 0.1 ml of 0.2 M TEMP and 0.1 ml of 10^{-4} M AF were lyophilized separately in order to avoid the decomposition of AF or TEMP by the concentrated alkali salts. Then the former residue was dissolved in 1 ml of deuterium oxide (D_2O) to make a 1/15 M phosphate buffer (pH 9.2), and the latter residue was dissolved in it.

Irradiation of Samples——Samples were irradiated with an ultraviolet (UV) lamp (Toshiba SHL-100 UV-2 type 100 W) from 3 cm distance.

Measurement of ESR Spectra——Spectra were recorded on a JEOL JES-3BS X spectrometer (X band) with 100 kHz field modulation (0.8 G). Relative intensity of 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPO) signals was expressed as the mean $\pm$ S.D. (mm) of the three peak-to-peak heights as described in the previous report.[4]

Photodynamic Action of Acridine Compounds on Yeast——A haploid yeast, *Saccharomyces cerevisiae* DP1 1B/517 (α, his_1, trp_1, ρ^+, ω^+, C^R), was grown for 18 h in YPD medium at 30 °C with shaking. Cells were washed with 1/15 M phosphate buffer (pH 7.0), sonicated to separate clumping cells and resuspended at a cell density of 10^8/ml in the buffer. Cells (10^6/ml) were incubated with 1—100 μM acridine compounds at 30 °C for 60 min in the dark. Aliquots of sensitized cell suspension were taken into Thunberg tubes and irradiated with fluorescent lamps (National 15 W × 4, 7 cm distance), while the remaining cell suspensions were kept in the dark as a control. Cells sensitized with AF, PF, AY, DAEA or AO were irradiated for 30 min, and those with A, Q or AA for 60 min. Cell suspensions, irradiated or unirradiated, were spread on plates of YPD medium after suitable dilution. After 3 d of incubation at 30 °C in the dark, the frequencies (%) of petite colonies were determined by the tetrazolium-overlay method[6] as described in the previous report.[2] Survivors were calculated from the number of colonies relative to the dark control.

Chemicals——Acriflavine was purchased from Aldrich. It is a 70:30 mixture of 3,6-diamino-10-methylacridinium chloride (euflavine) and 3,6-diaminoacridine (proflavine). Acridine orange and deuterium oxide were purchased from Merck, acridine hydrochloride and acridine yellow from Wako, quinacrine dihydrochloride and 9-aminoacridine hydrochloride from Nakarai, 6,9-diamino-2-ethoxyacridine lactate from Sigma, proflavine hemisulfate from Tokyo Kasei and 2,2,6,6-tetramethyl-4-piperidone from Aldrich. Authentic TEMPO was synthesized as described elsewhere.[7]

Results

TEMPO Production in AF Solution by Photo-Irradiation and Inhibition by the Addition of NaN_3

In order to confirm that the TEMPO production specifically depends on singlet oxygen, TEMPO production was compared under aerobic and anoxic conditions and in the presence of NaN_3. Each sample was taken into a capillary tube as described in Materials and Methods. The ESR spectrum of each sample was measured after photo-irradiation as described in the

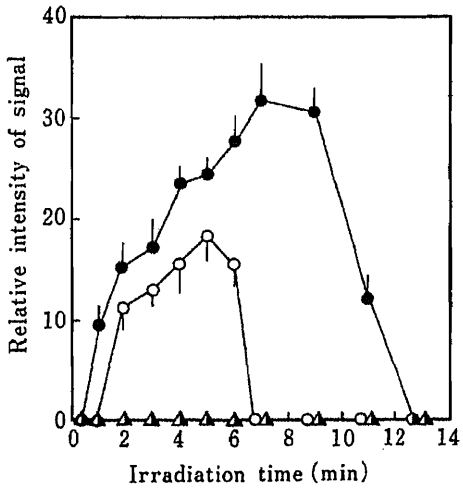

Fig. 1. Time Course of TEMPO Production by Photo-Sensitization of Acriflavine

Standard, D_2O, anoxic and NaN_3 samples were prepared as described in Materials and Methods. Each capillary was irradiated with a UV lamp from 3 cm distance. The ESR results are expressed as the mean $\pm$ S.D. (mm) of the peak-to-peak height. Standard sample, O—O; D_2O sample, ●—●; anoxic sample, △—△; NaN_3 sample, ▲—▲.

previous report.[4] Typical triplet signals of nitroxide radical (TEMPO) were detected in the irradiated aerobic sample. Relative intensity of TEMPO signals increased with increasing irradiation time but sharply decreased after 8 min, as shown in Fig. 1. However, TEMPO signals were not detected in the anoxic sample. Further, 50 mM NaN_3 completely inhibited the TEMPO production.

Enhancement of TEMPO Production in D_2O

TEMPO production by the photodynamic action of AF was determined in D_2O as described in Materials and Methods. The intensity of signals of TEMPO radical was higher and more long-lived in D_2O than in H_2O as shown in Fig. 1.

These results clearly indicated that TEMPO production is closely related to singlet oxygen production.

Comparison of the TEMPO Production Among Acridine Compounds

Time courses of the TEMPO production by 8 acridine compounds (Fig. 2) were compared. As shown in Fig. 3, all acridine compounds used here induced TEMPO production by photo-activation. AF and PF produced the most intense and long-lived ESR signals. The

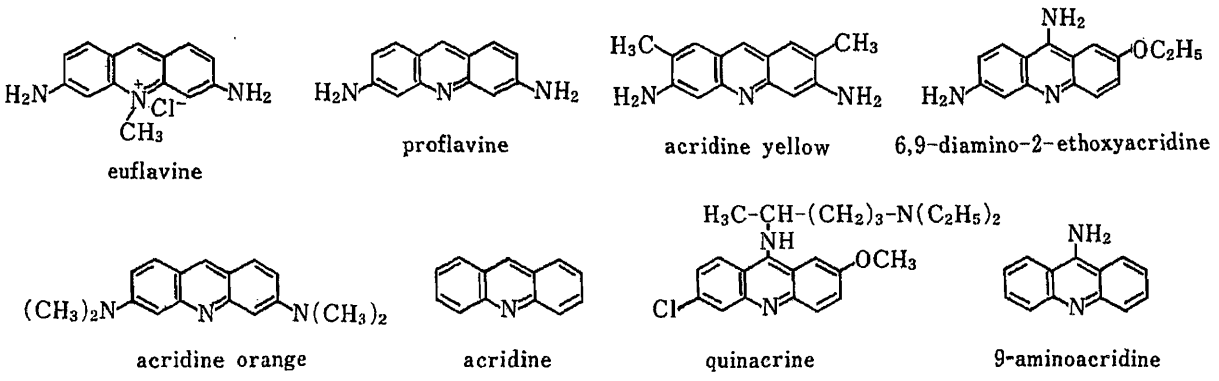

Fig. 2. Chemical Structures of the Acridine Compounds

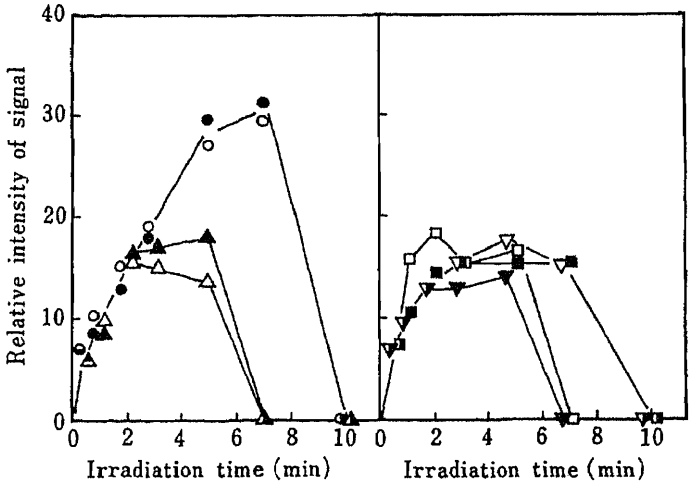

Fig. 3. Time Course of TEMPO Production by Photo-Sensitization of Acridine Compounds

Samples containing 2×10^{-2} M TEMP and 10^{-5} M acridine compound in 1/15 M phosphate buffer (pH 9.2) were irradiated with a UV lamp. Acriflavine (AF), ○—○; proflavine (PF), ●—●; acridine yellow (AY), △—△; 6,9-diamino-2-ethoxyacridine (DAEA), ▲—▲; acridine orange (AO), ▽—▽; acridine (A), ▼—▼; quinacrine (Q), □—□; 9-aminoacridine (AA), ■—■.

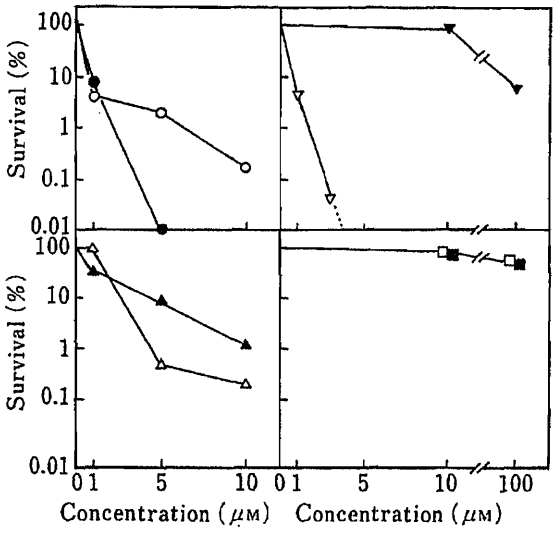

Fig. 4. Cell Inactivation by Photodynamic Action of Acridine Compounds at Various Concentrations

Yeast cells were incubated with acridine compounds in the dark and irradiated under fluorescent lamps. The values of cell survival (%) were calculated from the number of colonies relative to that of the dark control as described in Materials and Methods. Symbols are the same as in Fig. 3.

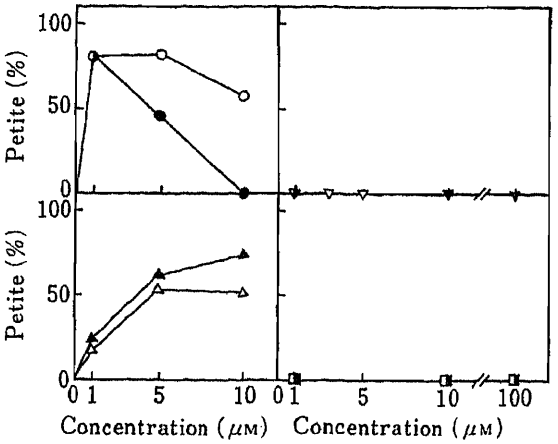

Fig. 5. Petite Induction by Photodynamic Action of Acridine Compounds at Various Concentrations

Yeast cells were treated as described in the legend to Fig. 4. The frequencies (%) of petite colonies were determined by the tetrazolium overlay method as described in Materials and Methods. Symbols are the same as in Fig. 3.

ESR signals produced by AO or AA were of average intensity, but were long-lived.

Cell Inactivation by Photodynamic Action of Acridine Compounds

As shown in Fig. 4, all acridine compounds induced photodynamic cell inactivation more or less. However, they could be divided into two groups in terms of their efficacy in cell inactivation. AF, PF, AY, DAEA and AO induced extensive cell inactivation, which depended on dye concentration, within 30 min of photo-irradiation. However, the activities of A, Q and AA were very low and were detected only at high concentration (100 μM) and after prolonged irradiation (60 min). The cell inactivation activities of 8 acridines were ranked as follows; AO > PF > AF = AY > DAEA ≫ A = Q = AA. Cell inactivation was not observed in any dark control.

Petite Induction by Photodynamic Action of Acridine Compounds

The difference between active acridines and inactive ones was clear-cut, as shown in Fig. 5. AF, PF, AY and DAEA induced petites remarkably after 30 min of photo-irradiation, while, A, Q, and AA did not even after 60 min of irradiation. Petite induction by PF was maximum at 1 μM and decreased with increasing concentration. Such a decrease of the induction rate at 10 μM was also observed in AF or DAEA photo-sensitization. Only the petite induction by AY was higher at 10 μM than at 1 μM. At high concentration, the dye molecules present in the solution, cytoplasm or mitochondrial matrix, or bound to membranes, may absorb light, so that less energy is available to activate the dye molecules.[2b,5]

None of the acridine compounds induced detectable petites without irradiation under such resting conditions.

Discussion

The ESR method for the detection of singlet oxygen is based on the assumption that

TEMP is specifically oxidized by singlet oxygen to TEMPO, which is a stable nitroxide radical whose characteristic signals are detectable by ESR measurement.

In this work, TEMPO production was confirmed to be an adequate ESR spectroscopic indicator of singlet oxygen production. Namely, TEMPO production was completely inhibited in the presence of NaN_3, which is an efficient quencher of singlet oxygen (Fig. 1).[8] Furthermore, TEMPO production was remarkably enhanced in D_2O (Fig. 1), in which the half-life of singlet oxygen is extended.[9]

The signal intensity of TEMPO is not necessarily proportional to the amount of singlet oxygen. However, if singlet oxygen is not produced, TEMPO will not be produced under our experimental conditions. The greater the amount of singlet oxygen produced, the greater the amount of TEMPO, and larger TEMPO signals should be produced. Therefore we may be able to judge the singlet oxygen-producing activity from the signal intensity of TEMPO, at least semi-quantitatively.

Using this method, the singlet oxygen-producing activities of 8 acridine compounds were compared (Fig. 3). All the acridine compounds were proved to have singlet oxygen-producing ability AF and PF, which are the most potent photo-sensitizers in both cell inactivation and petite induction (Figs. 4 and 5) produced the most intense TEMPO signals. A and Q, which had been believed to be ineffective in terms of cell-inactivating activity,[5] produced as much singlet oxygen as AY and DAEA, which effectively induce both cell inactivation and petite mutation. Reexamination of the photodynamic cell-inactivating activities of A, Q and AA (Figs. 4 and 5) revealed that those have the ability to inactivate yeast cells, but only at high concentration (100 μM) and after prolonged irradiation (60 min).

These results suggest that all acridine compounds used here have similar abilities to produce singlet oxygen but the distribution of each sensitizer in the cell and the interaction with each target may be different and be important determinants of the photodynamic biological activities of each photo-sensitizer. AF, PF, DAEA and AY, which have amino substituents at position 3 and/or 6, may be efficiently incorporated by yeast cells and intercalated into mitochondrial deoxyribonucleic acid (DNA). Photo-irradiation may activate these dyes and produce singlet oxygens which inactivate yeast cells and induce petite mutation. On the other hand A, Q and AA, which do not have an amino substituent at position 3 or 6, may not interact with cell components or be incorporated into mitochondrial DNA efficiently. AO which has dimethylamino substituents at the 3 and 6 positions is a well known photosensitizer[1a,8b,10] and was reported to be an effective dye for phototherapy for cancer.[11] In this work it inactivated yeast cells effectively (Fig. 4) but did not induce petite mutations (Fig. 5). The TEMPO-producing activity of AO was average, but the life time of TEMPO was long. These results suggested that AO is a potent singlet oxygen-producer and interacts with cell components, but is not accumulated in yeast mitochondria. We may be able to clarify the drug distribution within the cell on the basis of the specific biological effects of each photo-sensitizer.

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Isolation and Identification of Hypotensive Principles in Red-Mold Rice

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During an initial search for pharmacologically active principles in red-mold rice, two hypotensive compounds were isolated and identified. The active principles, A-3 and B-1, were fractionated and purified from the EtOH extract of red-mold rice prepared with *Monascus pilosus* through extraction with H_2O, Amberlite CG-120 column chromatographies and high-performance liquid chromatography on an ODS column. A-3 was identified as acetylcholine chloride by comparison with an authentic sample, and B-1, colorless needles of mp 203—205 °C, was proved to be identical with authentic γ-aminobutyric acid. Intravenously administered A-3 (0.5 μg/kg) and B-1 (250 μg/kg) produced potent depression of the carotid arterial blood pressure in spontaneously hypotensive rats, as did the corresponding authentic samples.

Keywords——red-mold rice; *monascus*; crude drug; blood pressure; hypotension; hypertension; hypotensive compound; acetylcholine; γ-aminobutyric acid; SHR

Monascus purpureus, *M. pilosus*, and *M. anka* (Eurotiaceae) are representatives of the *Monascus* fungi traditionally used in southern China, Formosa, Japan, Korea, Hong-Kong, *etc.* as a source of bright red pigments. Red-mold rice prepared by use of these fungi was first mentioned in Li Shih-chun's "Pen Chaw Kang Mu" (Li Shizhen, "Ben-Cao-Gang-Mu" in Chinese; an ancient monograph on Chinese herbal medicine), published in 1590. In this publication and another recent monograph,[1] manufacturing procedures for red-mold rice were described, in addition to the therapeutic activities of the rice. The red-mold rice has been used as a crude drug (Hong-Qu in Chinese; Beni-Kohji in Japanese) for "Huo-Xue-Hua-Yu (in Chinese)" (mobilization of blood circulation and treatment of stasis) in China and Formosa. Furthermore, diseases claimed to be cured by the red-mold rice included indigestion (Xiao-Shi in Chinese), bruising of muscles, dysentery, *etc.* Red rice wines fermented by the use of these fungi in China and Formosa differ in color, sweetness, and flavor. Fungi of the genus *Monascus* are also used as a source of food colorants in Japan and Formosa.

In our study on red-mold rice prepared with *M. pilosus*, we found two hypotensive principles in the rice, and isolated and identified them. In this paper we should like to present the details of this work.

Experimental

Materials——Red-mold rice was prepared as follows. Well-milled rice was immersed in water for 12 h, removed from the water and autoclaved for 30 min at 120 °C. *M. pilosus* IFO 4520 was inoculated into the cooked paddy rice and cultured for 8 d at 30 °C under aerobic conditions. The rice was dried at 40 °C to about 10% moisture content. Acetylcholine chloride, γ-aminobutyric acid (GABA) and Amberlite resins (Organo Ltd.) were purchased from

Nakarai Chemicals, Ltd., and Somnopentyl (sodium pentobarbital, Pitman-Moore Inc.) and amino acid standard solution from Wako Pure Chemical Ind., Ltd. Cholinesterase, acetyl (Type III from electric eel) was a product of Sigma Chemical Co. All other reagents and solvent were special grade products of Wako Pure Chemical Ind., Ltd.

Analytical Procedures——Acetylcholine was determined by the fluorometric method of Fellman[2] and carboxylic acid ester by the method of Hestrin.[3] Cholinesterase treatment was carried out by incubating the sample with the enzyme (0.5 unit) in 0.5 ml of saline (pH 8.0) at 37 °C for 10 min. Amino acid analysis was performed on a Hitachi 034 liquid chromatograph. Analytical and preparative high-performance liquid chromatography (HPLC) was performed on a Yanaco L-4000S instrument equipped with a Shodex RI SE-31 differential refractometer. A pre-packed column (Chemcosorb 7-ODS-H) was used. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Infrared (IR) spectra were run with a JASCO A-302 instrument. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a JEOL JNM-GX400 (400 MHz) spectrometer with D_2O as the solvent and the sodium salt of 3-(trimethylsilyl)-propionic acid-d_4 as an internal standard. Electron impact mass spectra (EI-MS) and accurate MS were taken with a JEOL JNS-DX300 mass spectrometer equipped with a direct inlet system at 30 eV, and field desorption (FD)-MS with the same mass spectrometer using carbon emitters under the following conditions: accelerating voltage, 3 kV and emitter current, 18—20 mA.

Extraction and Fractionation——Red-mold rice, usually 10 kg, was immersed in 4 volumes of EtOH for one week. The mixture was suction-filtered through Toyo filter paper No. 2 on a Buchner funnel and the filtrate was set aside. The residue was extracted twice with EtOH as above. The three filtrates were pooled and evaporated at 50 °C in a vacuum to leave the EtOH extract (141 g). The extract was suspended in 2 l of H_2O, and the suspension was mixed and shaken with 500 ml of AcOEt. The H_2O phase was washed with 500 ml each of AcOEt, five times, and with 500 ml each of BuOH, five times. The H_2O phase was evaporated at 50 °C in a vacuum to leave the H_2O fraction, which was dissolved in 28% AcOH and applied to an Amberlite CG-120 column (Type I, pyridine form, 5.0 × 25 cm). The column was washed with 2 l of 0.2 M pyridine–AcOH buffer (PA, pH 3.1), followed by elution with 2 l of 1.2 M PA (pH 4.9). The eluate with 1.2 M PA was evaporated at 50 °C in a vacuum and the residue was finally lyophilized to leave the 1.2 M PA fraction. Further purification of the active fraction was carried out by means of Amberlite CG-120 chromatography and HPLC.

Measurement of Arterial Blood Pressure (BP)——The animals used were 12- to 20-week-old male spontaneously hypertensive rats (SHR). The rats were anesthetized with pentobarbital (30 mg/kg, i.p.), and placed on a heating pad maintained at 37 °C to keep the body temperature constant. The right carotid artery and the left jugular vein were cannulated with polyethylene tubing for BP recording and sample administration, respectively. Mean BP was recorded by means of a pressure transducer, TP-200T, connected to a Polygraph system, RM-6000 (Nihon Koden). A test sample, dissolved in saline and adjusted to pH 6.5—7.0, was intravenously injected.

Results

Isolation of Hypotensive Principles

As shown in Table I, hypotensive activity during the extraction and fractionation of the active fraction from the red-mold rice was recovered in the H_2O fraction and 1.2 M PA fraction, successively. The 1.2 M PA fraction was fractionated by means of Amberlite CG-120 chromatography, as shown in Fig. 1. Hypotensive activity was divided into a Hestrin

Table I. Yield and Hypotensive Activity of Fractions Obtained from Red-Mold Rice

Fraction	Yield (mg) from red-mold rice, 10 kg	Hypotensive activity	
		Dose (mg/kg, i.v.)	Maximum decrease of mean BP (mmHg, mean ± S.E., $n=3$)
H_2O	56900	10	24 ± 4
1.2 M PA	3700	1	33 ± 4
A	17	0.25	60 ± 7
B	802	1	27 ± 3
A-1	4.8	0.05	59 ± 7
A-2	0.85	0.01	45 ± 4
A-3	0.15	0.0025	58 ± 5
B-1	300	0.25	30 ± 3

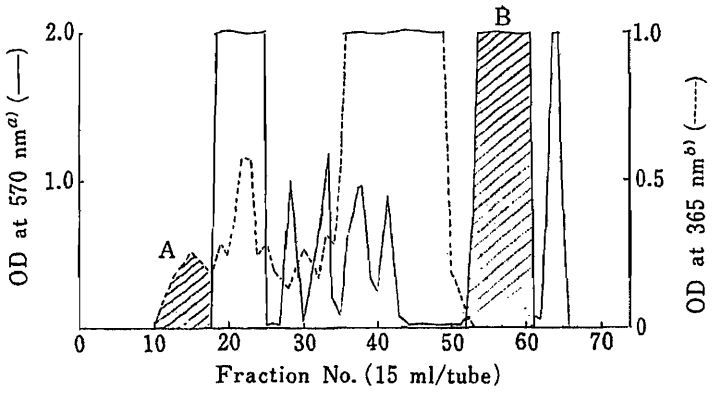

Fig. 1. Amberlite CG-120 Chromatography of 1.2 M PA Fraction

Column, 1.8 × 156 cm; eluant, 0.4 M PA (pH 4.6); flow rate, 100 ml/h; temperature, 55 °C. Shaded parts indicate fractions with hypotensive activity. *a*) A 30 μl aliquot was taken for ninhydrin reaction. *b*) A 100 μl aliquot was taken for Hestrin reaction.

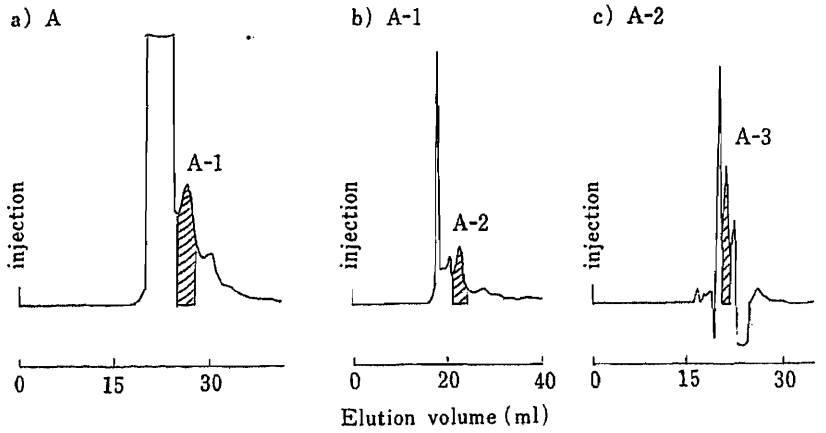

Fig. 2. Successive HPLC of A on Chemcosorb

Column, 1.0 × 30 cm; eluant and flow rate, a) 5% aq. MeOH at 3 ml/min, b) H_2O at 4 ml/min, c) 5% aq. MeOH–0.1% HCl at 3 ml/min; detection, refractive index. Shaded parts indicate fractions with hypotensive activity.

reaction-positive peak, A, which was eluted at the change from 0.2 M PA to 0.4 M PA, and a ninhydrin reaction-positive peak, B, which was eluted at a position corresponding to approximately 2 column volumes.

As shown in Fig. 2, the active fraction, A, was further purified by successive HPLC separations with three different eluant systems. Hypotensive activity was recovered in A-1, A-2 and A-3, successively, as shown in Table I. The purified A-3 was found to be homogeneous in HPLC analyses with two different eluant systems ($t_R = 5.6$ min with H_2O at 4 ml/min; $t_R =$ 7.5 min with 5% aq. MeOH–0.1% HCl at 3 ml/min). The yield of A-3 was 150 μg from 10 kg of the red-mold rice. Intravenously administered A-3 (2.5 μg/kg) showed a potent but transient hypotensive effect, as shown in Table I.

Another hypotensive fraction, B, obtained from Amberlite CG-120 chromatography was further purified by HPLC separation to afford colorless needles, B-1, as shown in Fig. 3. The active principle, B-1, was homogeneous in HPLC analysis ($t_R = 5.0$ min with H_2O at 4 ml/min) and in amino acid analysis, as shown in Fig. 4. The yield of B-1 was 300 mg from 10 kg of the rice. Intravenously injected B-1 produced a hypotensive effect which continued for a few minutes, as shown in Table I.

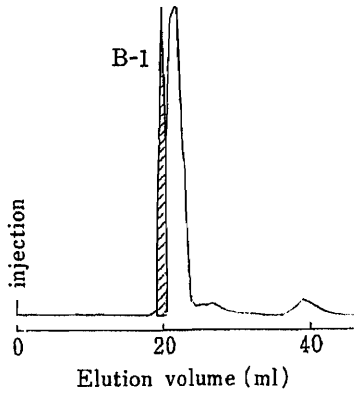

Fig. 3. HPLC of B on Chemcosorb

Column, 1.0×30 cm; eluant, H_2O; flow rate, 4 ml/min; detection, refractive index. The shaded part indicates the fraction with hypotensive activity.

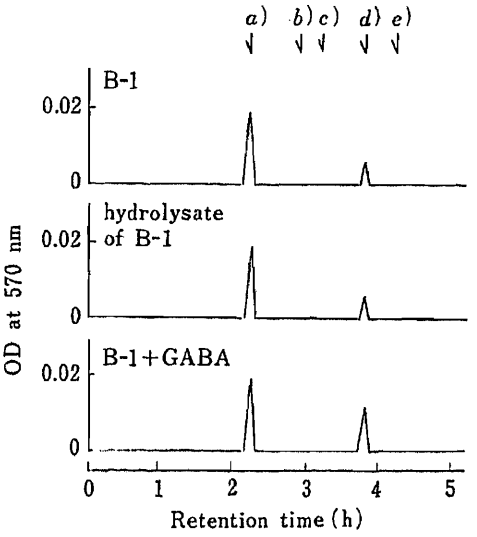

Fig. 4. Chromatograms Obtained by Ion Exchange Chromatography of B-1

Column, Hitachi custom #2611, 0.9×40 cm; eluant, stepwise with sodium citrate buffer (0.2 M, pH 3.25 to 0.2 M, pH 4.25 at 1 h 20 min, to 0.4 M, pH 4.40 at 2 h 45 min, then to 1.2 M, pH 4.80 at 3 h 45 min); flow rate, 60 ml/h; temperature, 55°C; detection, ninhydrin reaction; sample, B-1 6.25 μg, hydrolysate of B-1 (6 N HCl, 110°C, 22 h) equivalent to 6.25 μg of B-1, GABA 62.5 nmol, norleucine as an internal standard 62.5 nmol. The retention times of authentic samples are indicated by arrows: *a*) norleucine, *b*) β-alanine, *c*) γ-aminoisobutyric acid, *d*) GABA, *e*) ethanolamine.

TABLE II. Hypotensive Activity of A-3 Compared with Acetylcholine Chloride

Sample	Hypotensive activity	
	Dose (μg/kg, i.v.)	Maximum decrease of mean BP (mmHg, mean±S.E., $n=3$)
A-3	10	69± 9
	2.5	58± 5
	0.5	40± 6
A-3 treated with cholinesterase	2.5	—[a]
Acetylcholine chloride	10	74±10
	2.5	59± 8
	0.5	42± 5
Acetylcholine chloride treated with cholinesterase	2.5	—

a) Less than 5 mmHg.

Identification of Acetylcholine

A-3 was found to be acetylcholine in the chloride form.[4] ^{1}H-NMR δ: 2.13 (3H, s, -OCOMe), 3.21 (9H, s, $-\overset{+}{N}Me_3$), 3.73 (2H, m, $-CH_2\overset{+}{N}\leqslant$), 4.54 (2H, m, $-OCH_2$). FD-MS *m/z* (%): 329 $[(Me_3NCH_2CH_2OCOMe)_2^{2+}\cdot{}^{37}Cl^-$, 15],[5] 327 $[(Me_3NCH_2CH_2OCOMe)_2^{2+}\cdot{}^{35}Cl^-$, 42],[5] 146 $[(Me_3NCH_2CH_2OCOMe)^+$, 100]. The isolated active principle A-3 was found to be identical with authentic acetylcholine chloride by direct comparison. [^{1}H-NMR spectrum,

TABLE III. Hypotensive Activity of B-1 Compared with GABA

Sample	Hypotensive activity	
	Dose (mg/kg, i.v.)	Maximum decrease of mean BP (mmHg, mean ± S.E., $n=3$)
B-1	1	35±4
	0.25	30±3
	0.1	16±2
GABA	1	35±2
	0.25	31±1
	0.1	15±2

FD-MS and retention time ($t_R = 7.5$ min)]. Identity was also established by comparison of the biological properties of A-3 and authentic acetylcholine chloride, as shown in Table II. The two samples showed similar dose-dependent hypotensive activities which disappeared after pretreatment with cholinesterase.

Identification of GABA

B-1 was proved to be pure GABA. mp 203—205 °C (aq. EtOH). IR ν_{max}^{KBr} cm^{-1}: 1660 (sh), 1640 (sh), 1570, 1380. ^{1}H-NMR δ: 1.90 (2H, tt, $J=7.6$, 7.3 Hz, $-CH_2\underline{CH_2}CH_2-$), 2.28 (2H, t, $J=7.3$ Hz, $-CH_2\underline{CH_2}COOH$), 3.02 (2H, t, $J=7.6$ Hz, $-CH_2\underline{CH_2}NH_2$). EI-MS and accurate MS m/z (%): 103.0632 [M^+, Calcd for $C_4H_9NO_2$ (M) 103.0633, 42], 85 (100). B-1 was identical with an authentic sample of GABA on the basis of direct comparison [mixed mp 205—208 °C, IR (KBr), ^{1}H-NMR, EI-MS, accurate MS and retention times in HPLC (t_R = 5.0 min with H_2O at 4 ml/min) and on an amino acid analyzer ($t_R = 3.8$ h), as shown in Fig. 4. Further, B-1 and GABA showed the same hypotensive activity. (Table III).

Discussion

It is known that the *Monascus* genus produces pigments[6] such as rubropunctatin, monascorubrin and monascin, and enzymes[7] such as proteases and ribonucleases. Although red-mold rice has been used as a crude drug, it has not been established what chemical components are the active principles. In an initial examination of pharmacologically active principles in the red-mold rice prepared with *M. pilosus*, we have isolated and identified acetylcholine and GABA as hypotensive principles.

While GABA is widely distributed in microorganisms, the presence of acetylcholine is almost unknown except for *Streptobacterium plantarum*.[7] Acetylcholine is easily hydrolyzed on heating and exposure to alkaline solution to give choline and AcOH. The present conditions for isolation were thus not really appropriate for the isolation of acetylcholine. In fact, the acetylcholine content determined directly in the extract of the red-mold rice with cold 10% trichloroacetic acid[2] was 251 μmol (47.1 mg as the chloride form) per 10 kg of the rice, which was more than 300 times the content calculated from the yield of A-3.

It is well-known that acetylcholine and GABA are physiologically and pharmacologically active compounds. Acetylcholine, acting on muscarinic receptors of endothelial cells, stimulates the release of a substance that causes relaxation of the vascular smooth muscle, followed by systemic hypotension.[8] The action of acetylcholine is transient because it is easily hydrolyzed by cholinesterase in the body. Peripherally administered GABA produces comparatively prolonged hypotension, probably by exerting a neuromodulatory effect on cardiovascular function *via* peripheral actions which are influenced by type of anesthesia,

resting values of cardiovascular parameters, degree of activity of the sympathetic nervous system and catecholamine release from the adrenal medulla.[9]

Recently, Tsuji *et al.*[10] reported that chronic dietary administration of red-mold rice prepared with *M. pilosus* reduced the elevated blood pressure of SHR. The two active principles isolated here from the rice should account at least in part for the hypotensive effect of dietary administration of the rice, but this requires confirmation.

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20β-Hydroxysteroid Dehydrogenase of Neonatal Pig Testis: Localization in Cytosol Fraction and Comparison with the Enzyme from Other Species

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20β-Hydroxysteroid dehydrogenase (20β-HSD) was found in neonatal pig testes. The enzyme catalyzed the reduction of 17α-hydroxyprogesterone to 17α,20β-dihydroxy-4-pregnen-3-one with oxidation of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). Identification of 17α,20β-dihydroxy-4-pregnen-3-one as the product was achieved by thin layer chromatography, gas chromatography, high-performance liquid chromatography, nuclear magnetic resonance spectroscopy and mass spectrometry. 20β-HSD activity was contained in the cytosol fraction of testis but not the microsomal or the mitochondrial fraction. Furthermore, the enzyme required the presence of NADPH or reduced nicotinamide adenine dinucleotide (NADH) as a cofactor, though NADH was far less effective than NADPH. The cytosol fraction of neonatal pig testis contained a large amount of 20β-HSD in comparison with that of mature pig testis. The 20β-HSD activity was also found in the cytosol fraction of mouse, rat, dog and guinea pig testes, but the activities were very low.

Keywords——pig testis; cytosol; 20β-hydroxysteroid dehydrogenase; 17α,20β-dihydroxy-4-pregnen-3-one; NADPH; NADH

Stereospecific and reversible interconversions of hydroxyl and carbonyl groups of steroids are catalyzed by pyridine nucleotide-dependent hydroxysteroid dehydrogenases. The 20α-hydroxysteroid dehydrogenase (20α-HSD), which efficiently catalyzes the reduction of the 20-carbonyl group of pregnanes to the 20α(20*S*) product, seems to be present in various organs (liver, ovary, testis, adrenal, placenta) among several mammalian species. In particular, it has been purified and characterized from pig testis,[1] bovine testis[2] and rat ovary.[3]

On the other hand, it is known that 20β-HSD, which specifically catalyzes 20-carbonyl group reduction to the 20β(20*R*) product, is contained in the mycelium of *Streptomyces hydrogenans*.[4] In addition, it has been reported that 17α,20β-dihydroxy-4-pregnen-3-one was synthesized by the ovarian tissue of the teleosts, ayu[5] and amago salmon.[6] In mammalia, not only 17α,20α-dihydroxy-4-pregnen-3-one but also its 20β-epimer, 17α,20β-dihydroxy-4-pregnen-3-one, has been isolated from testes of young bulls.[7] That report suggests that 20β-HSD may be present in mammalian testicular tissue. In this paper, we wish to describe that 20β-HSD is localized more abundantly in cytosol fraction of neonatal pig testis.

Experimental

Chemicals——[4-^{14}C]-17α-Hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione, Lot No. 1319-151, 50 mCi/mmol) was purchased from New England Nuclear Corp., (Boston, Mass., U.S.A.) and its radiochemical purity was established by thin layer chromatography (TLC) before use. Non-radioactive 17α,20α-dihydroxy-4-pregnen-3-one and its 20β-epimer, other steroids, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and pyridine nucleotide cofactors [nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), oxidized form

($NADP^+$) and nicotinamide adenine dinucleotide, reduced form (NADH)] were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other chemicals used were of analytical grade.

Toluene solution containing 2,5-diphenyloxazole (0.4% w/v) and 2,2′-*p*-phenylene-bis-(5-phenyloxazole) (0.01% w/v) was used as the liquid scintillation fluid. All organic solvents were redistilled.

Tissue Preparation——Testes from neonatal (2 weeks old) pigs were obtained from a farm at castration. Testes from matured (1 year old) pigs were obtained from a slaughterhouse. Testes were also obtained from mice (ICR strain, 7 weeks old; C3H strain, 10 weeks old), rats (Wistar strain, 7 weeks old), guinea pigs (Hartley strain, 320—410 g), rabbits (New Zealand white strain, 2.2—2.5 kg) and dogs (matured crossbred). Human testis was obtained from a patient (70 years old) with prostate carcinoma.

The testes were weighed, decapsulated and homogenized in 0.15 M KCl–0.1 mM ethylenediaminetetraacetic acid (EDTA) with a loose-fitting glass-Teflon homogenizer. When the number of testes was large, a Waring blender was used for homogenization. The homogenate was centrifuged at $9000 \times g$ for 30 min, and the supernatant fluid was centrifuged at $105000 \times g$ for 60 min.

This supernatant fluid was used as the soluble fraction (cytosol). In order to obtain the subcellular fraction, neonatal pig testes were homogenized in 0.25 M sucrose–2 mM potassium phosphate buffer (pH 7.4)–0.1 mM EDTA. The mitochondrial fraction ($800 \times g$, 10 min–$9000 \times g$, 30 min; precipitate), the microsomal fraction ($9000 \times g$, 30 min–$105000 \times g$, 60 min; precipitate) and the cytosol fraction ($105000 \times g$, 60 min; supernatant) were obtained by conventional differential centrifugation.

The subcellular fractions obtained were stored at −20 °C until use. All of the above procedures were performed at 0—4 °C.

Incubation and Separation of Products——Radioactive steroid, [4-^{14}C]-17α-hydroxyprogesterone (0.01 μCi/20 nmol) was incubated with the subcellular preparations in the presence of NADPH (240 nmol) or NADH (240 nmol) in a total volume of 1 ml of potassium phosphate buffer (50 mM, pH 7.4) at 37 °C for 20 min.

All incubations were carried out in duplicate. After the incubation, 10 ml of CH_2Cl_2 was added to the incubation medium and the mixture was shaken vigorously. After removal of the aqueous fluid, the organic solvent extract was dried with anhydrous sodium sulfate, and evaporated under a nitrogen stream at 40 °C. The residue was applied to a TLC plate (Kodak; 13181 silica gel with fluorescent indicator), which was developed with benzene : acetone = 8 : 2 (v/v). Under these conditions, the *Rf* values of 17α-hydroxyprogesterone, 17α,20β-dihydroxy-4-pregnen-3-one and 17α,20α-dihydroxy-4-pregnen-3-one were found to be 0.66, 0.39 and 0.29, respectively. After radioautography (Fuji X-ray film, Rx), the relevant radioactive areas of the chromatogram were cut out and the ^{14}C radioactivity of each spot was measured with a liquid scintilation counter (Packard Tri-Carb 460).

For identification of 17α,20β-dihydroxy-4-pregnen-3-one as the product, non-radioactive 17α-hydroxyprogesterone (5.0 mg/1.25 ml of methanol) was incubated with the cytosol fraction (88 mg) of neonatal pig testis in the presence of an NADPH-generating system (5 μmol of $NADP^+$, 125 μmol of glucose-6-phosphate and 20 units of glucose-6-phosphate dehydrogenase) in 25 ml of potassium phosphate buffer (50 mM, pH 7.4) at 37 °C for 8 h. After preparative TLC, the main area corresponding to 17α,20β-dihydroxy-4-pregnen-3-one was cut out, and the steroid was eluted with $CH_2Cl_2 : CH_3OH = 1 : 1$ (v/v). The partially purified product was further purified by high-performance liquid chromatography (HPLC).

Enzyme Assay——The 20β-HSD and 20α-HSD activities were determined from the ^{14}C radioactivity present in the fractions corresponding to 17α,20β-dihydroxy-4-pregnen-3-one and 17α,20α-dihydroxy-4-pregnen-3-one respectively, after TLC as described above. The enzyme activities are corrected for recovery, which was calculated from the ratio of total ^{14}C counts of all areas of the thin layer plate to the ^{14}C counts of radioactive steroid added to the incubation medium as the substrate.

Analytical Method——Gas chromatography (GC) and HPLC were performed on Shimadzu GC-4CM PF and LC-6A machines, respectively.

The column for GC was SE-30(3%) on Chromosorb WAN DMCS (0.3 × 200 cm; temp, 270 °C, flow rate, 40 ml/min; detector, FID). The column for HPLC was Lichrospher Si-100 (0.4 × 25 cm) developed with ethyl acetate : hexane 3 : 7 (v/v) at the flow rate of 3 ml/min, and the peaks were monitored at 250 nm. The mass spectrum (MS) was measured on a JEOL TMS-D300 spectrometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum in $CDCl_3$ was measured on a JEOL JNM-FX100 instrument; chemical shifts are given in δ (ppm).

Protein concentration was measured by the method of Lowry[8] using bovine serum albumin as a standard.

Results

Identification of 17α,20β-Dihydroxy-4-pregnen-3-one

An aliquot of the purified product, 17α, 20β-dihydroxy-4-pregnen-3-one subjected to TLC, GC, and HPLC. The *Rf* values in TLC were 0.39 (benzene : acetone = 8 : 2, v/v) and 0.72 ($CHCl_3$: ethyl acetate : ethanol = 25 : 2 : 1, v/v). The retention times were 11.4 min (GC) and

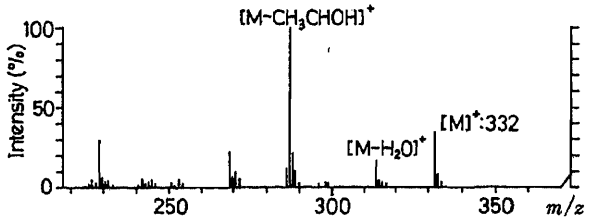

Fig. 1. Electron Impact Mass Spectrum of the Product, 17α,20β-Dihydroxy-4-pregnen-3-one

TABLE I. Subcellular Distribution of 20β-HSD of Neonatal Pig Testis

Subcellular fraction	Protein (mg)	20β-HSD		
		Specific activity (pmol/min/mg)	Total activity (nmol/min)	%
Cytosol	12120	64.9	786	93.5
Microsomes	2900	13.4	39	4.6
Mitochondria	1070	14.0	15	1.8

Decapsulated neonatal pig testes (223 g) were homogenized and separated to obtain subcellular fractions by conventional differential centrifugation as described under Experimental.

8.9 min (HPLC). Further, an aliquot of 17α,20β-dihydroxy-4-pregnen-3-one was subjected to ^{1}H-NMR and mass spectrometric analysis. The ^{1}H-NMR spectrum showed signals at δ 0.85 (3H, s, 18-CH_3), δ 1.19 (3H, s, 19-CH_3), δ 1.18 (3H, d, J=6 Hz, 21-CH_3), δ 4.02 (1H, J=6 Hz, 20-H) and δ 5.72 (1H, br, 4-H). The MS showed a molecular ion [M^+] peak at m/z 332, and fragment ion peaks at m/z 314 [$M-H_2O$]$^+$, m/z 287 [$M-CH_3CHOH$]$^+$ and m/z 269 (Fig. 1).

On the basis of these analytical data, it was confirmed that the product is 17α,20β-dihydroxy-4-pregnen-3-one. The stereochemical configuration of the C-20 hydroxyl group was estimated from the ratios [$M-H_2O$]$^+$/[M]$^+$ and [$M-CH_3CHOH$]$^+$/[M]$^+$ on MS of the 20α- and 20β-epimers.[9] The above analytical data were identical with those of authentic 17α, 20β-dihydroxy-4-pregnen-3-one.

Subcellular Distribution and Cofactor Requirement of 20β-HSD

More than 93.5% of the total activity of 20β-HSD was present in the cytosol fraction of neonatal pig testis, as shown in Table I. The microsomal and mitochondrial fractions contained only 4.6% and 1.8% of the total activity. The results suggest that most of the 20β-HSD is localized in the cytosol fraction.

Conversion of 17α-hydroxyprogesterone to 17α, 20β-dihydroxy-4-pregnen-3-one by 20β-HSD in testicular cytosol of neonatal pig required NADPH. However, NADH was far less effective than NADPH. In the case of NADPH as a cofactor, 43.3% of 17α-hydroxyprogesterone was reduced in 30 min, whereas only 11.1% was reduced in the case of NADH.

20β-HSD Activity in Testis from Other Species

The cytosol fraction of testicular tissue of other species also contained 20β-HSD activity. Specific activities are shown in Table II in terms of 17α,20β-dihydroxy-4-pregnen-3-one production from 17α-hydroxyprogesterone per min per mg of protein. 20β-HSD activities of other species were lower than that of mature pig testicular cytosol. In the case of pig testis, there was a marked difference in content of 20β-HSD between the neonatal and mature samples; 20β-HSD was contained more abundantly in neonatal pig testis than mature pig testis.

TABLE II. 20β-HSD and 20α-HSD Activities in the Testicular Cytosol Fraction from Various Animal Species

Species	Enzyme activities[a] (pmol/min/mg)	
	20β-HSD	20α-HSD
Pig (neonatal)[b]	64.9	23.9
Pig (mature)[c]	3.7	14.9
Mouse (ICR)	1.5	23.9
Mouse (C3H)	N.D.	33.0
Rat	1.1	15.3
Guinea pig	2.1	24.9
Rabbit	N.D.	24.7
Dog	1.4	13.0
Human (a patient with prostate carcinoma)	N.D.	8.6

a) The cytosol fraction (1.5—2.1 mg) was used as the source of 20β-HSD and 20α-HSD. *b*) Obtained at castration from immature (2 weeks old) pig. *c*) Obtained from adult (1 year) pig. N.D. = Not detected: The enzyme activity was below the limit of detection.

No correlation was found between the activities of 20α-HSD and 20β-HSD, as shown in Table II.

Discussion

17α-Hydroxyprogesterone was reduced not only to 17α,20α-dihydroxy-4-pregnen-3-one but also to 17α,20β-dihydroxy-4-pregnen-3-one by incubation with the testicular tissue of neonatal pig. The result shows that 20β-HSD is present in the testicular tissue, and most of its activity was found to be localized in the cytosol fraction.

20β-HSD of *Streptomyces hydrogenans* has been purified[10] and its properties have been clarified.[11] It requires NADH specifically and does not react with NADPH, whereas pig testicular 20β-HSD requires NADPH for conversion of 17α-hydroxyprogesterone to 17α,20β-dihydroxy-4-pregnen-3-one and NADH is far less effective than NADPH.

As shown in Table II, the testicular cytosol of mature pig and other species reduced the 20-carbonyl group of 17α-hydroxyprogesterone predominantly to the 20α product. However, reduction to the 20β product was predominant in the cytosol fraction of neonatal pig.

The finding that 20β-HSD activity of neonatal pig is higher than that of mature pig is of interest, since 17α,20β-dihydroxy-4-pregnen-3-one is one of the most potent inducers of oocyte maturation in several female teleosts,[12-14] and is related to the spermiation of salmonids.[15] As a preliminary to investigating the possibility that this steroid has a hormonal function at the stage of maturation, the purification and characterization of 20β-HSD are in progress in our laboratory.

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Separation and Identification of Amine–Carbohydrate Reaction Product in Aqueous Solution

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Drugs with a primary aromatic amino moiety such as procaine, procainamide, and *p*-aminobenzoic acid (PABA) are capable of reacting with various reducing sugars containing hexoaldoses and pentoaldoses. The amine–carbohydrate reaction products can be separated from the parent drugs by high performance liquid chromatography (HPLC) analysis. The formation and dissociation profiles of the products were followed by HPLC and ultraviolet spectroscopy. The reaction products formed in aqueous solution were not association complexes, but chemical reaction products, which were identified as glycosylamines or *N*-glycosides, by comparison with authentic samples obtained by the condensation of amines with carbohydrates in methanol. An X-ray analysis of one of the glycosylamines, 2-deoxy-*N*-*p*-carboxyphenyl-D-ribosylamine, was carried out, and the absolute conformation and configuration in the crystalline state were determined.

Keywords——*p*-aminobenzoic acid (PABA); procaine; procainamide; glucose; 2-deoxyribose; amine–carbohydrate condensate; glycosylamine; HPLC analysis; UV spectroscopy; X-ray analysis

In the pharmaceutical field, a number of carbohydrates have been widely used as diluent bases (expanders), binders, disintegrators, taste-modifying agents, and so on. Moreover in many parenteral solutions, various kinds of carbohydrates are prescribed as a main component, and many other drugs are often dissolved in such parenteral solutions for clinical use. Thus, numerous studies on the interaction of drugs with carbohydrates have been done. Over thirty years ago, from the anomalous changes in optical rotation of glucose solutions containing procaine, Cannell suggested that condensation occurred to form *N*-(D-glucosyl)procaine.[1] Later, Kaito[2] and Ikeda[3] also reported the *N*-glucoside formation of procaine in aqueous glucose solutions. In these studies the reaction was followed by polarimetry,[1] by paper chromatography,[2] and by chemical analysis of the parent compound after solvent extraction.[3] Recently, the interaction of drugs with carbohydrates has been examined again, with the use of modern analytical methods.[4] Kirschenbaum *et al.* observed a decrease of the procainamide concentration in 5% dextrose solution to less than 90% of the original concentration after 24 h of storage.[5] Baaske *et al.* reported similar phenomena and suggested the direct reaction of glucose with procainamide to yield one or more entirely new compounds.[6] Thereafter, Das Gupta examined the procainamide–glucose reaction product by high performance liquid chromatography (HPLC) analysis and described this reaction as complex formation.[7]

In a study of the effect of carbohydrate on the stability of local anesthetics, we also found new reaction products of procaine, procainamide, and *p*-aminobenzoic acid (PABA) with several reducing sugars by HPLC analysis. The amine–carbohydrate reaction products separated chromatographically from the parent drugs were expected to be glycosylamines or

N-glycosides,[8] as reported in the previous studies.[1-3] The present study was performed in order to determine whether the new reaction products are glycosylamines or not.

Experimental

Materials——Procaine (PC) was supplied by Fuso Pharmaceutical Industries Co., Ltd., and procainamide by Midori-Juji Co., Ltd. PABA was purchased from Wako Pure Chemical Industries Co., Ltd., and recrystallized from methanol–water several times. D-Glucose was provided by Meito Industries Co., Ltd., and other carbohydrates (D-galactose, D-ribose, and 2-deoxy-D-ribose) were purchased from Nakarai Chemical Co., Ltd. Deionized distilled water and acetonitrile of chromatographic grade were used for the preparation of the eluent for HPLC analysis. Methanol was of chromatographic grade and other chemicals were of reagent grade (used without further purification).

Preparation of *N*-*p*-Carboxyphenyl-D-glycosylamines (*N*-PABA-GA)——*N*-PABA-GA were prepared by condensation of PABA with D-glucose, D-galactose, D-ribose, and 2-deoxy-D-ribose (dRib). They were obtained by reacting PABA with equimolar amounts of the above carbohydrates suspended in methanol for 0.5—3 h at 20—60 °C until the attainment of complete miscibility. On cooling and/or evaporation of the solvent under reduced pressure at below 40 °C, solids or crystals were separated. They were crystallized from methanol twice or three times, and dried *in vacuo* at 40—60 °C. In the case of 2-deoxy-*N*-*p*-carboxyphenyl-D-ribosylamine (*N*-PABA-dRib), large single crystals were obtained, and subjected to X-ray analysis. The physical properties of several *N*-PABA-GAs are summarized in Table I. Elementary analysis of *N*-PABA-dRib did not give complete agreement of the experimental values with the calculated ones due to the presence of residual methanol of solvation (Table I). Moreover the *N*-PABA-GAs were not stable, and turned brown during storage at room temperature for several months. Eventually they decomposed completely to black, tarry substances, as reported by Ellis and Honeyman.[9]

Formation and Dissociation of Amine–Carbohydrate Reaction Product——Formation of the Reaction Product: Reaction of an amine with a carbohydrate was initiated by mixing them in phosphate buffer (0.1 M, pH 7.0, ionic strength 0.3), in a 10 ml centrifuge tube with a stopper. Initial concentrations of amine and carbohydrate were 0.5—2.0 mM and 0.05—0.25 M, respectively. The reaction mixture was kept at 40±0.1 °C in a water bath under thermostatic control. Aliquots of 5—10 μl of the solution were taken at appropriate intervals, and subjected to HPLC analysis.

Dissociation of the Reaction Product: The amine–carbohydrate reaction is a reversible equilibrium system, and the equilibrium was easily disturbed by simple dilution. After 2.5—10 fold dilution of the equilibrated mixture (2 mM amine+0.25 M carbohydrate), dissociation or degradation of the reaction product was followed by HPLC analysis. The diluting solution was the same buffer solution as that used in the formation procedure. The diluted samples were kept at 40±0.1 °C, and at appropriate intervals the changes of the composition were followed by HPLC. In order to follow the dissociation process by spectroscopy, 40—50 fold dilution of the equilibrated mixture was necessary. The spectral measurements were carried out under ambient conditions.

For examination of the hydrolysis of authentic glycosylamine, a definite amount of glycosylamine dissolved in methanol (3.93 mM) was mixed with a buffer solution of the desired pH value, and the hydrolytic behavior was

Table I. Physical Data for *N*-*p*-Carboxyphenyl-D-glycosylamines (*N*-PABA-GA)

Glycosylamine	Mol. formula (M_r)	mp (°C)	$[\alpha]_D^{20}$ (°)[a]	λ_{max} (nm)[b]	Analysis (%) Found (Calcd)		
					C	H	N
N-*p*-Carboxyphenyl-D-glucosylamine	$C_{13}H_{19}NO_7$ (301.29)	165—170 (dec.)	−77	270	52.3 (52.2	5.9 5.7	4.7 4.7)
N-*p*-Carboxyphenyl-D-galactosylamine	$C_{13}H_{19}NO_7$ (301.29)	150—155 (dec.)	−112	271	52.0 (52.2	5.7 5.7	4.6 4.7)
2-Deoxy-*N*-*p*-carboxyphenyl-D-ribosylamine	$C_{12}H_{15}NO_5$ (255.24)	140—145 (dec.)	+20	271	55.9 (56.9	6.4 6.0	5.3[c] 5.5)
N-*p*-Carboxyphenyl-D-ribosylamine	$C_{12}H_{15}NO_6$ (269.24)	147—151 (dec.)	+67	270		—	

a) Optical rotation was measured in methanol and extrapolated to zero time. *b*) UV absorption was measured in phosphate buffer solution (pH 7.0) immediately after dissolution. *c*) The disagreement may have arisen from residual methanol of solvation. If 1/2 MeOH remains in one 2-deoxy-*N*-*p*-carboxyphenyl-D-ribosylamine molecule, the experimental values would agree well with the calculated ones (C : H : N = 55.8 : 6.3 : 5.2).

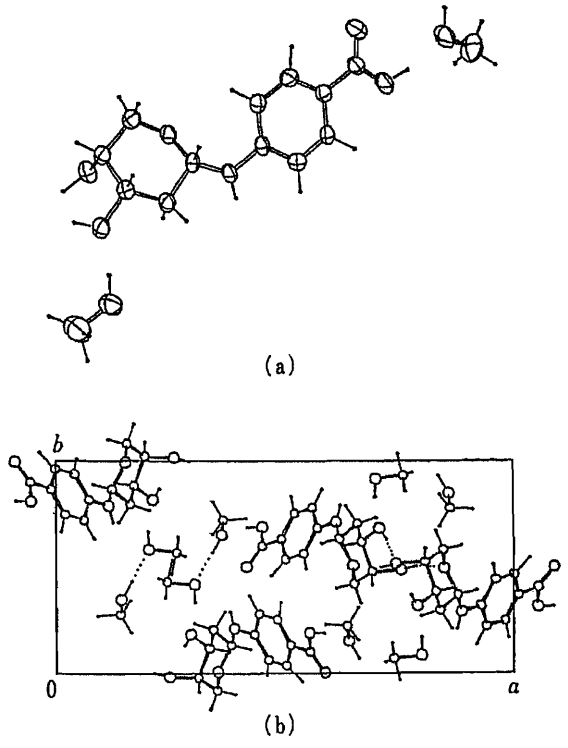

Fig. 1. X-Ray Analysis of 2-Deoxy-*N*-*p*-carboxyphenyl-D-ribosylamine (*N*-PABA-dRib)

a) Perspective view of the *N*-PABA-dRib molecule with two molecules of methanol of solvation.
b) Projection along the *c*-axis.

followed by HPLC and ultraviolet (UV) spectroscopy as described above.

HPLC Analysis——An HPLC apparatus (model LC-5A) equipped with a UV detector (model SPD-2A) and with a photodiode array detector (model SPD-MIA) (Shimadzu Co.) was used, and the wavelength was set at 270 or 280 nm. The stationary phase used was CHEMCOSORB 7 DPH (15 cm long × 4.6 mm i.d., Chemco Co.), and the mobile phase consisted of acetonitrile–sodium acetate buffer (50 mM, pH 3.5) which was varied in composition from 97 : 3 to 84 : 16 according to the combination of amine and carbohydrate. The flow rate was 1.5 or 1.8 ml/min. A short column (3 cm long × 4.6 mm i.d.) packed with CHEMCOSORB 7 DPH was used to guard the main column. All chromatographic operations were carried out under ambient conditions.

Physical Measurements——Melting points were determined on a micromelting point apparatus (Yanagimoto Co.). UV absorption spectra were measured with model 100-60 and model 556 spectrophotometers (Hitachi Co.) under ambient conditions. Optical rotations of authentic *N*-PABA-GA in methanol were followed by with a model PM-201 polarimeter (Union Co.) at 20±0.1 °C, using a 10 cm cell with a stopper.

X-Ray Analysis of *N*-PABA-dRib——Single crystals of *N*-PABA-dRib were prepared by slow crystallization from methanol. Since the crystals were not stable and lost transparency rapidly on filtration, a crystal was sealed in a glass capillary in the solvent of recrystallization (methanol) for X-ray analysis. The crystal was orthorhombic, space group $P2_12_12_1$ with the unit cell dimensions $a=21.490$ (4), $b=9.999$ (2), $c=7.448$ (2) Å, and $D_{calcd}=1.317\ g\cdot cm^{-3}$ for $Z=4(C_{14}H_{23}NO_7$ or $C_{12}H_{15}NO_5\cdot 2(CH_3OH)$, M_r 317.34); eight solvent (methanol) molecules were included in a unit cell. A crystal with dimensions of 0.5 × 0.4 × 0.2 mm was used for data collection. The intensity data were measured on a Rigaku AFC-5RU diffractometer with graphite-monochromated CuK_α radiation ($\lambda=1.54178$ Å), using the $\omega-2\theta$ scan method at the rate of $16^\circ\cdot min^{-1}$. The structure was solved by the direct method using MULTAN 78, and refined by the full-matrix least-squares method. The final *R*-value was 0.057 for 1365 observed reflections. All computations were performed on a FACOM M382 in the Data Processing Center of Kyoto University, using the KPPXRAY programs.

As shown in Fig. 1, the 2-deoxy-D-ribose sugar ring has a pyranose chair form in the $^1C_4$ conformation, and the anomeric carbon atom has the β-configuration. Thus this compound exists as 2-deoxy-*N*-*p*-carboxyphenyl-β-D-ribopyranosylamine in the crystalline state.

Results and Discussion

Separation of Amine–Carbohydrate Reaction Product

It is well-known that PC is easily hydrolyzed to PABA and diethylaminoethanol in weakly alkaline solutions. The hydrolysis can also occur in aqueous carbohydrate solutions.

After partial hydrolysis of PC in 0.25 M dRib solutions, an aliquot was subjected to HPLC analysis. A typical chromatogram is shown in Fig. 2. Five peaks were detected in the following order of separation: PABA–dRib reaction product, PABA, PC–dRib reaction product, PC, and *p*-nitrophenol (internal standard).

Retention times of the reaction products were shorter than those of the parent drugs, due to higher hydrophilicity. Although Das Gupta failed to detect the PABA–glucose reaction product,[7] we found the product and also PABA–dRib reaction product, as described below. Like PC and PABA, procainamide could also react with dRib and glucose. Thus the amine–carbohydrate reaction product detected in HPLC analysis seems not to be specific to the combination of procainamide and glucose as reported by Das Gupta[7a] and others.[5-6] From previous data on the formation of *N*-(glycosyl)procaine with reducing sugars,[1-3] the reaction products found in the present study were suggested to be the condensation products of amines with carbohydrates.

Formation and Dissociation of the Reaction Product

In Fig. 3 the mode of formation of the reaction product between PABA and dRib is shown in terms of successive chromatograms after the addition of PABA to 0.25 M dRib

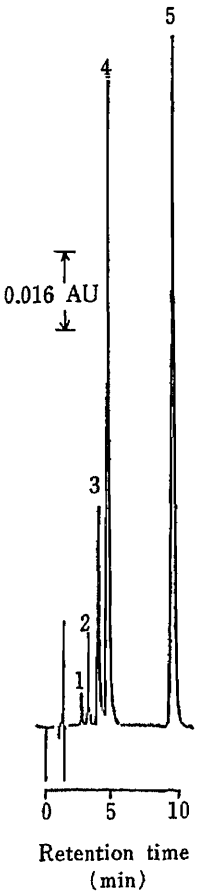

Fig. 2. Chromatographic Separation of the Amine–Carbohydrate Reaction Product

Peaks 1—5 denote PABA–dRib reaction product, PABA, PC–dRib reaction product, PC and *p*-nitrophenol (internal standard), respectively.

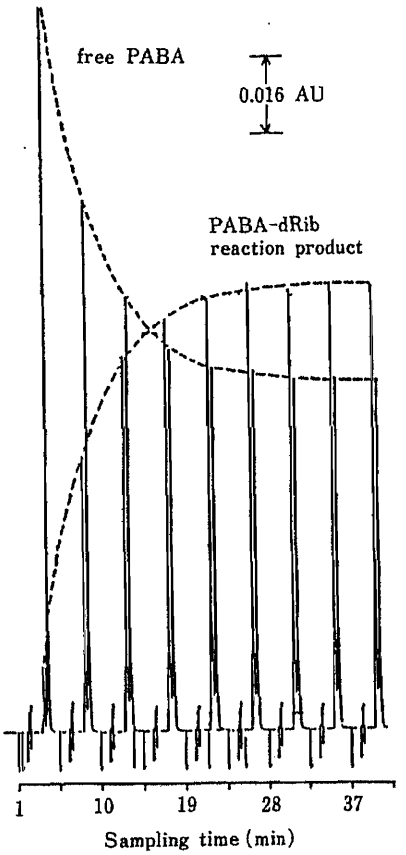

Fig. 3. Successive Chromatograms of the PABA–dRib System after Mixing of the Components at pH 7.0 and at 40 °C

The reaction mixture was composed of PABA (1.83 mM), dRib (250 mM), and phosphate buffer (100 mM, pH 7.0). The descending and ascending dotted lines, which connect the elution peaks, indicate the changes of the relative amounts of free PABA and PABA–dRib reaction product in the medium, respectively.

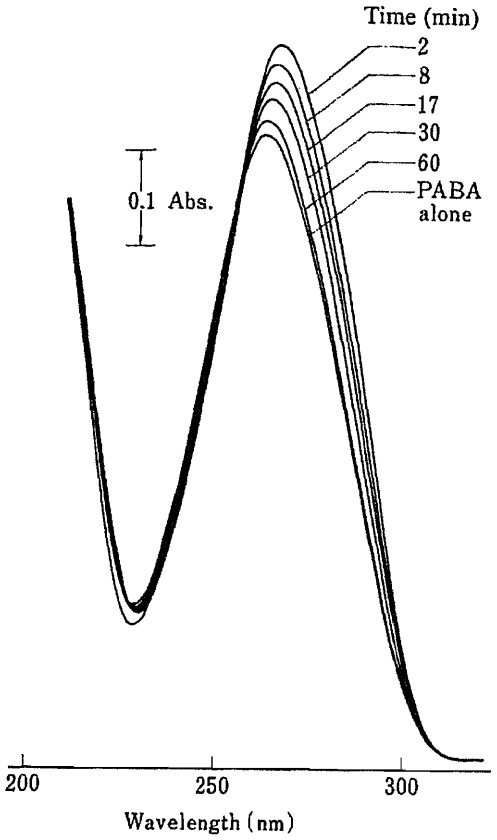

Fig. 4. UV Spectral Changes Due to the Dissociation of PABA–dRib Reaction Product at pH 7.0

UV spectral changes of PABA–dRib system were recorded after 40-fold dilution of the equilibrated PABA–dRib mixture (1.83 mM PABA, 0.2 M dRib, 0.1 M phosphate buffer, pH 7.0).

solutions (pH 7.0, 40 °C). A rapid increase of the reaction product occurred concomitantly with a decrease of free PABA, as indicated by the dotted lines. The reaction had almost reached equilibrium after 30 min under the present conditions. The peak height ratio of the product to free PABA at equilibrium changed linearly with the concentration of dRib at a constant concentration of PABA. That is, the ratio increased with increment of the relative concentration of the carbohydrate.

On the other hand, when the equilibrium mixture of PABA–dRib was diluted with buffer solutions (same components as the reaction medium), a rapid dissociation of the product and establishment of a new equilibrium were observed by HPLC. Furthermore the process of dissociation could be followed by UV spectroscopy, as shown in Fig. 4. The PABA–dRib reaction product gradually dissociated into free PABA and dRib after 40-fold dilution at room temperature. After 60 min, the dissociation was almost complete and nearly the same spectrum as that of an equimolar concentration of standard PABA solution (4.57×10^{-5} M) was obtained. Thus, in highly diluted PABA–dRib mixtures only a small amount of the reaction product remains. These spectral changes showed two characteristic features: one is the hypsochromic shift of the absorption maxima by about 4—5 nm in the course of the dissociation, and the other is the existence of the isosbestic point at around 255 nm.

When a large excess of dRib exists compared to PABA, formation and dissociation of the product can be treated as a reversible 1st-order reaction; these results will be reported elsewhere. Reaction of PABA with glucose also occurred under the same conditions in the case of the PABA–dRib system, but the rate was much slower.

The above-mentioned chromatographic and spectroscopic behavior of amine–carbohydrate mixtures might be interpreted in terms of complex formation, as suggested in some recent reports.[5-7] However, in general, complex formation based on weak intermolecular forces is a rapid process in homogeneous systems under usual conditions. Thus, many

organic complexes cannot be separated from their solutions as definite compounds, and they are often difficult to detect by usual chemical and physical means.[10] In contrast, the present amine–carbohydrate interaction proceeds slowly (Figs. 3 and 4), and in the case of PABA–glucose mixture the reaction rate is much slower than in the PABA–dRib system under the same conditions, as mentioned above. Moreover, the rate of the reaction is markedly dependent on temperature, as also found by Baaske *et al.*[6] and Ikeda,[3] which suggests the interaction to be a chemical reaction with a fairly large activation energy. Thus, it is reasonable to consider the present amine–carbohydrate interaction as being not a complex formation, but a reversible chemical reaction, like the mutarotation of reducing sugars.[11]

Identification of Amine–Carbohydrate Reaction Products

If, as discussed above, the products are chemical reaction products of amines and carbohydrates, such as glycosylamines or *N*-glycosides,[8] their formation corresponds to the condensation of amines with carbohydrates, and their dissociation, to the hydrolysis of glycosylamines. Several kinds of *N*-PABA-GAs were synthesized by condensation of PABA with various reducing sugars in methanol (see Table I). Confirmation of the structure of *N*-PABA-dRib was obtained by X-ray analysis (see Fig. 1 and Experimental). As can be seen in Fig. 1, a covalent bond is formed between the anomeric carbon of dRib and the amino nitrogen of PABA, and the interatomic distance of the C–N single bond is estimated to be 1.437 Å, as compared to the calculated value of 1.47 Å.[12] Thus, at least in methanol, the primary aromatic amine and reducing sugar molecules are covalently combined with elimination of water, resulting in the formation of the glycosylamine.

A typical condensation reaction with elimination of water is observed in the reaction of aldehydes with primary amines,

$$RCHO + R'NH_2 \rightleftharpoons \left[\begin{array}{c} OH \\ | \\ R\text{–}C\text{–}H \\ | \\ NHR' \end{array} \right] \rightleftharpoons RCH{=}NR' + H_2O$$

where the intermediates and the resulting products are called carbinolamines and imines or Schiff bases, respectively. However, most imines are fairly unstable and difficult to isolate. Nevertheless, in many organic and biological reactions, it is well known that imines play an important role as intermediates. Thus, if the present reaction does involve the condensation of aromatic amines with reducing sugars, the acyclic form of the sugar will presumably be involved in the first step of the reaction, and it is predicted that the formation of the glycosylamine may proceed through a carbinolamine and an immonium ion [R–CH=NHR'].[13] Therefore, because of the instability of imines or Schiff bases, the formation of the glycosylamines can be expected to proceed also in aqueous solutions, as reported by Ikeda.[3]

Next, we examined whether or not the UV absorption spectra, the chromatographic behavior, and the mode of hydrolysis of the authentic glycosylamine are consistent with those of the amine–carbohydrate reaction products as determined by HPLC analysis.

The hydrolytic behavior of authentic glycosylamine was observed by HPLC and UV spectroscopy, and the results agreed well with those obtained by diluting the equilibrated amine–carbohydrate mixture. Details of the kinetic studies will be published elsewhere. During the hydrolysis of *N*-PABA-dRib at pH 7.0, the spectral changes were similar to those found on dissociation of the reaction product (Fig. 4). Figure 5 shows the spectral changes during *N*-*p*-carboxyphenyl-D-glucosylamine (*N*-PABA-Glu) hydrolysis at pH 1.26. Remarkable spectral changes were observed with a slight hypsochromic shift of the absorption maxima. The marked changes may arise from the protonation of the primary aromatic amine in such a strongly acidic region. As can be seen in Fig. 5, *N*-PABA-Glu was completely hydrolyzed to free PABA and D-glucose after 16 h under the conditions used.

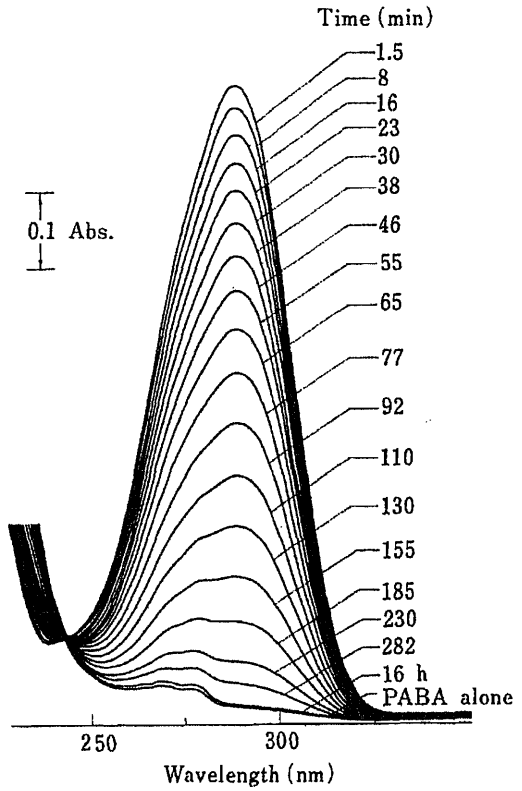

Fig. 5. UV Spectral Changes Accompanying the Hydrolysis of *N*-*p*-Carboxyphenyl-D-glucosylamine (*N*-PABA-Glu) at pH 1.26

Authentic *N*-PABA-Glu was dissolved in aqueous hydrochloric acid–potassium chloride solution (pH 1.26, ionic strength 0.3), to give a final concentration of 3.20×10^{-5} M.

The retention times of the authentic samples were in good agreement with those of the reaction products found in aqueous amine–carbohydrate mixtures. A chromatogram for the PABA–glucose system is shown in Fig. 6(A); the retention time of the PABA–glucose reaction product (peak 1, t_R 2.4 min) agreed well with that of authentic *N*-PABA-Glu (peak 3), though the nature of the minor peak just before peak 3 is unknown. Moreover, by using an HPLC apparatus equipped with a photodiode array detector, UV spectra of the three compounds (peaks 1—3) were recorded at the respective elution peaks during an HPLC run, as shown in Fig. 6(B). In addition to the similarity of the spectra of the reaction product and authentic glucosylamine over a wide wavelength region, the absorption maximum of the former (λ_{max} 293 nm) agreed well with that of the latter, and the bathochromic shift of λ_{max} by about 4 nm was also found, as anticipated from the dissociation profiles of the PABA–dRib and PABA–glucose systems (see Figs. 4 and 5).

From the above results and discussion, the reaction products found chromatographically in aqueous solutions containing reducing sugars and drugs with a primary aromatic amine moiety can be identified as the glycosylamines. Therefore, the procainamide–glucose complex detected by HPLC[5-7] seems not to be an association complex, but a chemical reaction product (a glucosylamine). In a mixture of procainamide and glucose, we also found a new peak in HPLC attributable to *N*-(D-glucosyl)procainamide.

In spite of the long-standing finding of the formation of glycosylamines by reaction between primary aromatic amines and reducing sugars in aqueous solution the reaction has not been well understood, as is clear from the recent reports.[5-7] It is worth noting that several *N*-(substituted phenyl)-D-glycosylamines have been synthesized and tested for ability to inhibit the growth of several malignant animal tumors[14] and the replication of cultured B 16 melanoma cells.[15] Although in these studies the stability of the glycosylamines in the cell culture medium was not taken into account, some of these compounds were evaluated as new bioactive compounds independent of the parent amino compounds. It seems necessary to

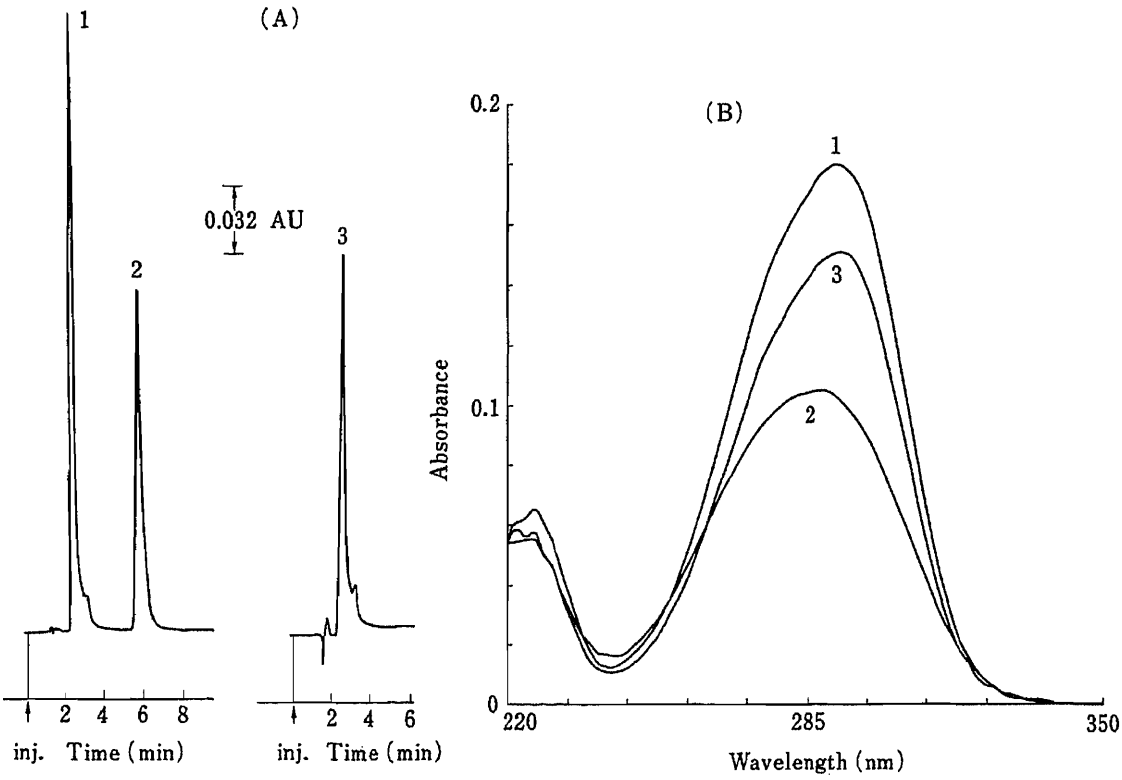

Fig. 6. HPLC Chromatograms and UV Absorption Spectra of PABA, PABA–Glucose Reaction Product, and Authentic *N*-*p*-Carboxyphenyl-D-glucosylamine (*N*-PABA-Glu), Obtained with a Photodiode Array Detector

(A) HPLC chromatograms; peaks 1—3 denote PABA–glucose reaction product, free PABA, and authentic *N*-PABA-Glu, respectively.

(B) UV spectra of the above three compounds, recorded at the respective elution peaks during an HPLC run by using a photodiode array detector. Curves 1—3 denote, PABA–glucose reaction product, PABA and authentic *N*-PABA-Glu, respectively.

investigate in detail the formation and dissociation of glycosylamines, because the pharmaceutical and biological significance of these phenomena is still not sufficiently clear.

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Release Characteristics of Nifedipine Sustained-Release Granules *in Vitro* and in Healthy Subjects

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The release characteristics of nifedipine sustained-release granules, composed of ethylcellulose, hydroxypropylmethylcellulose and corn starch, were examined *in vitro* as well as in healthy subjects. The release of nifedipine from the granules *in vitro* was not first-order, but a linear relationship up to about 40% release was obtained based on the Higuchi equation. The release rate was not strongly influenced by pH, stirring speed, surfactant or ionic strength. These granules were administered to healthy subjects and the plasma levels of nifedipine were compared with those after administration of nifedipine soft gelatin capsule. Plasma levels following the administration of the granules were prolonged as compared with those in the case of the soft gelatin capsule, and plasma levels of about 13 ng/ml at 12 h post-dosing were detected. The known problem of marked inter-subject variability of the plasma levels was not encountered after administration of the sustained-release granules, presumably due to the multiple-units dosage form and the established low sensitivity of the drug release rate to the *in vitro* environment, *i.e.*, pH, stirring speed, surfactant and ionic strength.

Keywords——nifedipine; sustained-release granules; release characteristics; oral administration; healthy subject; plasma level; inter-subject variability

Introduction

The intensity of the pharmacologic or toxic effect of a drug is considered to be related to the concentration of the drug at the receptor sites, which are usually located in the tissue cells. In practice, the plasma drug level is generally employed for monitoring the course of therapy, since most of the tissue cells are richly perfused with tissue fluids or plasma. The drug concentration is determined by the rates of absorption, distribution and clearance. The effect of a drug may be controlled by reducing its rate of absorption, by delaying the rate at which it is inactivated, or by retarding its excretion.[1] One method to prolong the plasma drug level is to employ a sustained-release formulation.[2]

In preparing sustained-release products, it is desirable to avoid the use of special apparatus such as a coating pan or a fluidized bed coating machine, and techniques such as microencapsulation, on grounds of convenience and expence. In the present study we chose nifedipine as a model compound. The extent of bioavailability of nifedipine is low when the drug is administered orally in a crystalline form because of poor water-solubility.[3] Several water-soluble formulations have been developed,[4] but the elimination half-life of nifedipine after administration of these formulations is only 3.43 h.[4b] It is, therefore, necessary to prolong the plasma levels in order to maintain the clinical effect. Recently some sustained-release formulations have been developed.[5] We also previously formulated two kinds of sustained-release granules; one with pH-dependent release and the other with pH-independent

release.[6] The pH-independent release granules were superior to the pH-dependent release granules with respect to prolonging the effective plasma levels and minimizing the inter-subject variability when administered to rabbits.[6]

In the present study the effects of stirring speed, surfactant and ionic strength (μ) on the release rate of nifedipine from the pH-independent release granules were examined *in vitro* to investigate the release characteristics. Furthermore, the pH-independent release granules were administered to healthy subjects and the plasma levels after administration of the granules were compared with those after nifedipine soft gelatin capsule.

Experimental

Materials——Ethylcellulose (100 cps, Wako Pure Chemical Industry, Osaka, Japan), hydroxypropylmethylcellulose (HPMC; 50 cps, Sigma Chemical Co., St. Louis, Mo. U.S.A.), corn starch (Hoei Yakuko, Osaka, Japan), nifedipine soft gelatin capsule (Adalat, Bayer) were used. Crystalline nifedipine (lot I34A, Kanebo Ltd., Osaka, Japan) was a gift. All other chemicals were of reagent grade.

Methods——All experiments were carried out in a dark room, because of the high sensitivity of nifedipine to light.[7]

Preparation of Granules——One gram of nifedipine, 3 g of ethylcellulose and 3 g of HPMC were dissolved in ethanol–dichloromethane (1 : 1, v/v) and then 8 g of corn starch was added with agitation by a magnetic stirring bar in a jacketed beaker connected to a thermostated water bath (Iuchi, Racom-Ace HT-01C). A slurry with suitable toughness was obtained by evaporating the solvent while maintaining the water bath at 70 °C, and then the product was forced through a 35-mesh sieve. The resultant cylindrically shaped initial materials clung to the sieve surface even after passing through the pores. After drying the initially formed materials at 50 °C, the sieve was scraped with a spatula. A fraction of granules with a size of between 32-mesh and 35-mesh was obtained, in this way.

Release Studies——The JPX paddle method[8] was employed for investigating the release rates from the granules. A cylindrical releasing vessel in a constant temperature water bath was used. The paddle was located at a depth of 25±2 mm from the bottom. The granules (75 mg) were dispersed in 500 ml of various kinds of medium: distilled water; distilled water containing 0.1 or 1% (w/v) polysorbate 80; 0.1 N HCl (pH 1.2, μ=0.100 M); 0.1 N HCl containing 1% sodium chloride (pH=1.1, μ=0.272 M); 0.05 M phosphate buffer (pH=7.0, μ=0.110 M); 0.1 M phosphate buffer (pH=7.0, μ=0.222 M), at 37±0.5 °C. The paddle was rotated at 150 rpm. In addition, rotation rates of 50 and 250 rpm were also used to examine the effect of stirring rate on the release rate. Samples (5 ml) were removed at suitable intervals and filtered through membrane filters with a pore size of 0.45 μm (Toyo Roshi Co., Ltd.). The solutions were analyzed spectrophotometrically against a blank at 238 nm. Fresh medium (5 ml) was added to the dissolution vessel immediately after each sample was removed to maintain the original volume. Triplicate runs were made in each study.

Solubility Studies——Saturated solutions of nifedipine were prepared in each dissolution medium and equilibrated at 37±0.5 °C in a thermostated water bath for 24 h. Each sample was filtered through the same type of membrane as used in the release studies. After suitable dilution with the same medium, these samples were spectrophotometrically analyzed against a blank at 238 or 340 nm. Triplicate runs were made in each study.

Content of Drug in Granules——The granules (30 mg) were dissolved in 20 ml of ethanol–dichloromethane (1 : 1, v/v) by shaking for 30 min and then centrifuged at 1500 *g* for 5 min. The supernatant was appropriately diluted with ethanol–dichloromethane (1 : 1, v/v) and spectrophotometrically analyzed against a blank at 340 nm. Triplicate runs were made in each study.

Administration of Drug——Four healthy male subjects (age range 21—32 years) participated in the present study. The study was designed in a randomized crossover fashion with at least a 14-d interval between doses. Each subject received orally 10 mg of nifedipine in a soft gelatin capsule or 20 mg of nifedipine in sustained-release granules with 100 ml of tap water at 9 : 00 a.m. after overnight fasting. About 5 ml of blood was obtained immediately before drug administration (time 0) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 9 and 12 h post-dosing. A cannula was inserted into a forearm vein and this was used for blood withdrawal for the first 4 h. After that, blood was collected from a forearm vein by means of a syringe. The blood specimens were centrifuged and the separated plasma samples were stored at −20 °C with protection from light until assay was done. The subjects remained supine for the first 2 h. Beverage was permitted during the study except in the first 4 h. The subjects were only allowed to take food at 4 and 10 h post-dosing.

Assay of Nifedipine in Plasma——Nifedipine in all samples was assayed according to the high performance liquid chromatography (HPLC) method of Miyazaki *et al.*[9] In this study, 1 ml of plasma was used. Methanol (100 μl) and acetonitrile (3 ml) were added to 1 ml of plasma in a brown test tube, and agitated with a vortex mixer. After centrifugation at 1500 *g* for 5 min, 3 ml of the supernatant was transferred into a brown test tube containing 1 ml of distilled water, and then 4.5 ml of acetone–chloroform mixture (1 : 1, v/v) was added. The mixture was shaken for

10 min and then centrifuged at 1500*g* for 5 min. The aqueous layer was aspirated off, and 5 ml of the organic layer was transferred into a brown test tube, and evaporated to dryness in a centrifugal evaporator (Model RD-21, Yamato Scientific, Tokyo, Japan) at 45 °C for 30 min. The residue was dissolved in 100 μl of the mobile phase containing butamben as an internal standard (2 μg/ml), and 20 μl of the solution was injected into the HPLC system. The chromatographic conditions were the same as previously reported.[9] The wavelength for the assay of nifedipine was 238 nm at 0.0025 AUFS.

Statistical Analysis——Equality of variances and the significance differences in the peak plasma levels (C_{max}), the times to the peak plasma levels (t_{max}) and areas under the concentration–time curves ($AUC_{0-12\,h}$) between capsule and granules were tested by means of the F and *t* tests, respectively.

Results and Discussion

We previously reported that the release rate of nifedipine from the pH-independent release granules could be controlled by altering the polymer ratio of HPMC–ethylcellulose used as a binder, and also that the release curves were not influenced by the pH of the dissolution medium.[6] The mechanism of drug release from the granules presumably involves the formation of a hydrated zone of HPMC on the surface of the granular matrix.[10,11] This would be the first step in the formation of a transport channel. Part of the drug would be diffused through the hydrated zone and be released into the medium while the remainder would be liberated when the hydrated zone dissolved. Altering the HPMC–ethylcellulose ratio in the polymer composite mixture would affect the release rate markedly.[10] When there is a low content of HPMC in the granular matrix, a comparatively slow release rate might be expected, due to the support provided by the hydrophobic ethylcellulose. In the present study, further investigations of the release characteristics from this sustained-release granular matrix were carried out. The content of nifedipine in the granules was 95.9±3.70% (mean±S.D.; $n=7$).

Wagner[12] showed that most sustained-action dosage forms for which release-time data are available in the literature released their contained drug into fluids in the *in vitro* test at pseudo- (or apparent-) first-order rates. It has become rather common practice to plot data derived from dissolution rate studies on conventional tablets and capsules in conformity with first-order kinetics. The percent drug remaining in the granules is plotted on the logarithmic scale of semilogarithmic graph paper against time in hours on the abscissa. The lack of linearity over the entire range in our case indicates that the process is not first-order. Therefore, an attempt was made to determine whether the drug release could be described by a diffusion equation proposed by Higuchi.[13] The plot gave a linear relationship up to about 40% release. The remainder is released more slowly, presumably due to greater occlusion of the drug by the insoluble ethylcellulose matrix[14] or failure to maintain the sink condition.

We also considered the effects of factors such as stirring speed, wetting and ionic strength on the release rate *in vitro*. The release rate was not much influenced even when the stirring speed was increased from 50 to 250 rpm. This indicates that the diffusion in the stationary layer at the outer wall of the granules was not rate-limiting. Furthermore, the effect of polysorbate 80 as a surfactant on the release rate was examined at 150 rpm. The solubilities of nifedipine at 37 °C in distilled water containing 0%, 0.1% and 1% (w/v) polysorbate 80 were found to be 11.0, 26.6 and 172.7 μg/ml, respectively (Table I). However, the release rates were less influenced by addition of polysorbate 80. It is interesting that solubilization and wetting by polysorbate 80 had little effect on the release rate despite the poor water-solubility of nifedipine. Since wetting depends markedly on surface tension, whereas the release rates were not correlated with surface tension, wetting was not a rate-limiting step for these processes. Similar results have been reported by Weintraub and Gibaldi,[15] and Schott *et al.*[16]

We also examined the effect of ionic strength on the release rate at 150 rpm. The solubility of nifedipine was decreased by about 15% with increment of the ionic strength from

TABLE I. Solubilities of Nifedipine in Various Media at 37 °C

Dissolution medium	Solubility (μg/ml)
Distilled water	11.0±0.38
Distilled water containing 0.1% (w/v) polysorbate 80	26.6±0.83
Distilled water containing 1% (w/v) polysorbate 80	172.7±2.02
0.1 N HCl (μ=0.100 M)	11.0±0.46
0.1 N HCl containing 1% NaCl (μ=0.272 M)	9.4±0.40
0.05 M phosphate buffer, pH 7 (μ=0.110 M)	9.4±0.12
0.1 M phosphate buffer, pH 7 (μ=0.222 M)	8.5±0.12

Each result is a mean value±S.D. (n=3).

TABLE II. Pharmacokinetic Parameters of Nifedipine after Administration of Soft Gelatin Capsule and Sustained-Release Granules

Parameter	Capsule	Granules	t test	F test
C_{max} (ng/ml)	240.8[a]±44.32 (148.8—332.2)	55.9±3.13 (47.9—62.9)	—[b]	U.E.V.[d]
t_{max} (h)	0.9[a]±0.36. (0.5—2.0)	2.4±0.55 (1.5—4.0)	NS[c]	E.V.[e]
AUC_{0-12h} (ng·h/ml)	454.3[a]±92.61 (246.8—664.0)	340.6±20.15 (287.5—372.1)	NS[c]	U.E.V.[d]

Each result is a mean value±S.E.M. (n=4). *a*) Corrected for the dose by multiplying by 2. *b*) Significant at $p<0.05$. *c*) Not significant at $p>0.05$. *d*) U.E.V., unequal variances at $p<0.05$. *e*) E.V., equal variances at $p>0.05$.

0.100 to 0.272 M in 0.1 N HCl (Table I). In addition, the solubility of nifedipine was decreased by about 10% with increment of the ionic strength from 0.110 to 0.222 M in the phosphate buffer (pH 7) (Table I). The release rates, however, were less influenced by these differences in ionic strength. Similar results were reported by Baggesen and Bechgaard[17]: the release rates of propoxyphene hydrochloride from pellets were not much influenced by ionic strength between 0.097 and 0.133 M. These results indicate that the pH-independent release granules prepared in the present study might be relatively impervious to factors such as pH, peristaltic movement, bile, or ionic strength of the GI fluid in the alimentary tract.

The mean plasma nifedipine levels of the sustained-release granules were higher over the 2—12 h period than those in the case of the soft gelatin capsule. The pharmacokinetic parameters for the two formulations are not directly comparable since the dose of nifedipine administered as granules was double the dose in the capsule. However, dose proportionality studies of nifedipine established linear kinetics.[5a,6,18] Thus, after correcting for the dose by multiplying C_{max} and AUC_{0-12h} by 2 for the capsule, these parameters were analyzed statistically (Table II). The peak plasma level for the granules was significantly lower ($p<0.05$) than that for the capsule (Table II). The extent of bioavailability of the granules was about 75% of the capsule, which was similar to that found in rabbits previously. The t_{max} value for the granules was larger than that for the capsule (Fig. 2), but there was no statistically significant difference (at $p>0.05$) in AUC_{0-12h} or t_{max} values between the two formulations. When the pharmacokinetic parameters were compared for the two formulations for unequal variances, the C_{max} and AUC_{0-12h} values after administration for the granules were significantly smaller ($p<0.05$) than those of the capsule (Table II). This smaller inter-subject variability may be attributed to the following factors. (1) The granular formulation which is a type of multiple-units dosage form[19] would be dispersed quickly in the

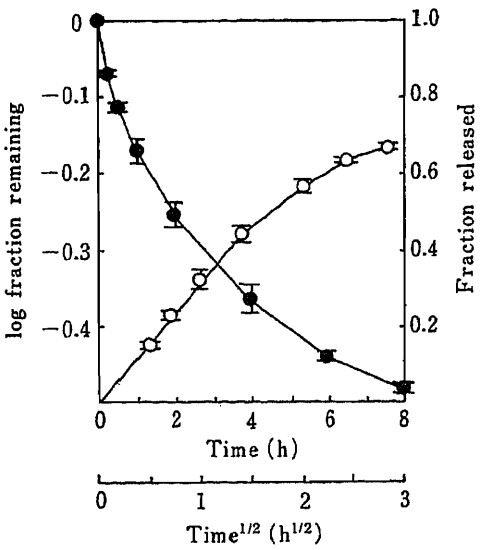

Fig. 1. Comparison between log Remaining Fraction–Time Plot and Fraction Released–Square Root of Time Plot of Data from Nifedipine Sustained-Release Granules

(●), log fraction remaining *versus* time; (○), fraction released *versus* square root of time. Each point represents a mean value±S.D. ($n=3$).

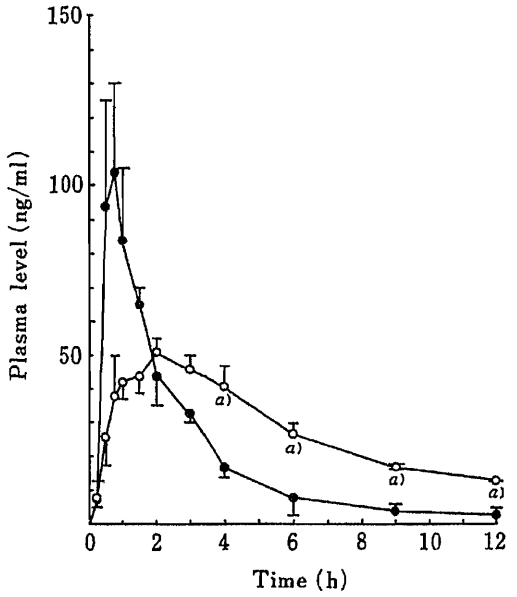

Fig. 2. Mean Plasma Nifedipine Profiles after Oral Administration of Two Formulations

(●), soft gelatin capsule containing 10 mg of nifedipine; (○), sustained release granules containing 20 mg of nifedipine. Each point represents a mean value±S.E.M. ($n=4$). *a*) $p<0.05$.

stomach and scattered through the small intestine, where they would be distributed widely.[20] Thus, inter-subject variability caused by differences in the transit time are minimized with such formulations. (2) The sustained-release granules prepared in the present study would be less influenced by factors such as pH and ionic strength in the GI fluid, peristaltic movement, or bile, based on the results obtained from the release studies *in vitro*.

The hypotensive effect of nifedipine in hypertensive patients was related to the plasma level[21] and the minimum nifedipine level required for the effect was 10—15 ng/ml.[5b,c,22] Thus the patients should receive capsules every 6 h (total: 40 mg/d) based on the results shown in Fig. 5. However, the granular formulation would be expected to be effective even if the patients receive it every 12 h (total: 40 mg/d). The plasma levels at 4, 6, 9 and 12 h after administration of the granules were significantly higher ($p<0.05$) than those after the capsule (Fig. 2), though the dose of nifedipine administered as granules was double the dose in the capsule. A plasma level of about 13 ng/ml at 12 h post-dosing was detected after administration of the granules. If two nifedipine soft gelatin capsules (equal to the dose of the sustained-release granules) are administered to the subjects, high peak plasma levels might produce severe side effects. The slower release of nifedipine from the granules may minimize the side effects, which are a result of high plasma levels.[23] Furthermore, it is easy to adjust the dose of the pH-independent release granules to correspond to the severity of the disease, but difficult to do so with a commercial sustained-release tablet[5a-c] or controlled-release capsule which contains rapid release and slow release granules.[5d]

In conclusion, a higher plasma level was detected over the 2—12 h period, and no great inter-subject variability was noted after oral administration of the nifedipine sustained-release granules prepared in the present study. Twice-daily dosing should be sufficient for therapeutic effectiveness. Thus, this formulation is expected to improve the compliance of patients.

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Effects of Concentration and Degree of Polymerization on the Rheological Properties of Methylcellulose Aqueous Solution

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Rheological studies were carried out on aqueous solutions of five methylcellulose (MC) preparations, with degrees of polymerization (DP) between 330 and 1020. Comparison of the intrinsic viscosity of MC with that of sodium carboxymethylcellulose (CMC) of similar DP indicated that MC molecules were relatively compact coils when they were present as isolated coils in dilute solution. However, the Huggins constants (k_1') of MC samples were higher than those of CMC samples, suggesting that intermolecular interaction of MC was larger than that of CMC at low concentration. The second virial coefficients of MC samples in dilute solution, determined by ultracentrifugation analysis, were divided at about DP 500 int two groups, low and high DP samples. A similar tendency was observed in the rheological properties of concentrated MC solution. It is suggested that the increase in DP was accompanied with an increase not only in molecular size but also in intermolecular interaction of MCs in solution. Interesting rheological characteristics were observed in high DP samples, particularly at concentrations higher than a critical concentration. The dynamic viscoelastic behavior of these samples depended significantly on both DP and concentration.

Keywords——methylcellulose; sodium carboxymethylcellulose; second virial coefficient; intrinsic viscosity; Huggins constant; steady state viscosity; dynamic viscoelasticity

A number of polysaccharide solutions show characteristic hydrodynamic behavior such as viscoelasticity or gel-like properties. This behavior is attributed to physical entanglement of molecules and/or chemical intermolecular interaction, forming a network structure in solution. There are many reports on the rheological properties of various polysaccharides. For a random coil polymer such as sodium carboxymethylcellulose (CMC) in the presence of NaCl, it was reported[1] that the intermolecular interaction was dependent on polymer concentration.

Methylcellulose (MC) in this study is a nonelectrolyte carbohydrate polymer, and it is well known that its aqueous solution shows a remarkable viscoelasticity. This property of MC solution is utilized in various practical fields such as medicine, foods, and cosmetics. A pronounced change in viscoelasticity at about 1.0% (w/v) concentration was found by Kuroiwa and Nakamura,[2] suggesting changes in the interaction between molecules at this concentration. However, as the practical concentration ranges are 1.0—2.0% (w/v), *i.e.*, the concentrated solution region, there have been few studies on the hydrodynamic properties over a wide concentration range from dilute to concentrated solutions, or on the effect of degree of polymerization (DP) on the interaction between MC molecules. In this study, the effects of concentration and DP on the rheological properties of MC aqueous solution were investigated.

Experimental

Materials——The following five commercial MC preparations were used: methyl cellulose (Wako Pure Chemical Industries, Ltd.) 25, 50, 400, 1500, and 4000 cP [these values are average viscosity of 2% (w/v) aqueous solution at

20 °C; methoxyl content, 27.2±0.6%]. MC was uniformly dispersed in hot water at about 80 °C; cold water was then added while stirring, and the dispersion was cooled gradually. The solution was allowed to stand at 4 °C overnight to dissolve the polymer completely. Two commercial CMC preparations (Tokyo Kasei Kogyo Co.; DP, *ca.* 500 and 1050; degree of esterification, 0.76±0.03) were used. CMC was dissolved in 0.15 M NaCl solution at about 37 °C. All experimental solutions were allowed to stand at room temperature overnight before measurements.

Distribution of Methoxyl Groups in MC——Gas chromatography–mass spectrometry (GC-MS) (JEOL JMS-D300 mass spectrometer coupled with a JGC-20K gas chromatograph) was used to evaluate the distribution of methoxyl groups in MC samples. After hydrolysis with 2 N hydrochloride for overnight at 100 °C, a sample was reduced with $NaBH_4$ and acetylated with acetic anhydride–pyridine solution (1 : 1, v/v). The solution was evaporated, dried over P_2O_5 *in vacuo*, then dissolved in $CHCl_3$.

Ultracentrifugation——Ultracentrifugation experiments were performed with a Hitachi UCA-1 analytical ultracentrifuge equipped with a schlieren optical system using an RH-60 rotor. All runs were performed at 20 °C. For this measurement, 0.05 M KBr solution was used as the solvent. Schlieren photograms of sedimentation cells or equilibrium cells were read with a microcomparator (Nikon model 6C).

Equilibrium ultracentrifugation as proposed by Yphantis[3] was performed to determine the weight-average molecular weights (M_w) of MC samples. In order to determine the partial specific volume (0.72 $ml \cdot g^{-1}$) of MC samples, a pycnometer was used. Equilibrium measurement was performed with a 4-channel cell at 12290 rpm with four concentrations ranging from 0.075 to 0.3% (w/v). In order to determine the initial concentration, a synthetic boundary-type center piece cell and a rotor speed of 6520 rpm were used. The DP of an MC sample was evaluated from M_w and the degree of substitution (DS) of methoxyl groups by GC-MS analysis, and the results are listed in Table II.

Sedimentation coefficients (s) of MC samples were determined at 60000 rpm in the concentration range from 0.05 to 0.3% (w/v). A single sector cell was used. The intrinsic sedimentation coefficient (s°) was obtained by extrapolating a plot of reciprocal sedimentation coefficient against concentration to zero concentration.

High-speed sedimentation equilibrium experiments (meniscus depletion method)[4] were performed with a 3-channel cell at 14400 rpm (MC 25 and 50) or 13310 rpm (MC 400, 1500 and 4000), using 0.1% (w/v) MC solutions. The point near the meniscus in the cell at the equilibrium state was assigned zero concentration, then the concentration at each point from this point to the bottom of the cell was obtained. The thermodynamic second virial coefficient was estimated from the slope of a plot of reciprocal apparent molecular weight at each point against concentration.

Dilute Solution Viscosity——A capillary viscometer (Cannon–Manning semimicro, No. 100) was used for determination of the intrinsic viscosity ($[\eta]$) and Huggins constants, k_1' and k_2' (Eq. 1),[5]

$$\eta_{sp}/C=[\eta]+k_1'[\eta]^2C+k_2'[\eta]^3C^2 \qquad (1)$$

where C is concentration ($g \cdot dl^{-1}$). Measurements were carried out at 37 °C. For MC solution, two solvent systems were used: aqueous solution (good solvent) expected to favor extended chains, and 50% ethanol solution (poor solvent) expected to favor comparatively compact coils. CMC in 0.15 M NaCl solution was also measured.

Viscosity with Stationary Flow——A cone-plate or a coaxial-cylinder type rotating rheometer (Shimadzu, RM-1, equipped with a reduction gear, RDG-1) was employed. The shear rate available ranged from 7.35×10^{-3} to 7.50×10^{2} s^{-1}. An apparent viscosity at a given shear rate was calculated from the shear rate and the observed shear stress. The shear rate dependence of viscosity was measured for each MC sample over the concentration range of 0.1—3.0% (w/v) at room temperature (21±1 °C).

For many solutions of small molecules (Newtonian liquids), shear rate increases linearly with increasing stress. In contrast, for most polymer solutions the apparent viscosity decreases with increasing shear rate; they are shear-thinning, and Newtonian behavior is observed only at low shear rate where η_0, the "zero-shear" viscosity can be established.[6] Specific and reduced viscosities were calculated for each solution by evaluating relative viscosity ($\eta_{rel}=\eta_0/\eta_s$, where η_s is the viscosity of solvent). Then, "zero-shear" intrinsic viscosity was determined by the use of Eq. 1 with lower concentration solutions.

Dynamic Viscoelasticity——By oscillatory rotation of the plate or the outer cylinder, dynamic viscoelasticity was determined with the same equipment. The angular frequency employed ranged from 2.90×10^{-2} to 3.48 $rad \cdot s^{-1}$. The rotation angle of the plate or the outer cylinder and the twist angle of the cone or the inner cylinder were recorded with an X–Y plotter. From the hysteresis loop obtained, the storage modulus or the dynamic elasticity (G') and the loss modulus (G'') were calculated.[7] The measurements were carried out with MC solution ranging from 1.0 to 3.0% (w/v) at room temperature (21±1 °C).

Results and Discussion

Distribution of Methoxyl Groups

GC-MS analysis was performed to evaluate the distribution of methoxyl groups in five

MC samples employed. Seven partially methylated glucitol acetates were observed. The contents (%) which were evaluated from the peak intensities of the acetyl group peaks ($m/z=43$) are given in Table I. Overall, the distributions of methoxyl groups in the five MC samples were similar. This indicated that the five samples differed only in degree of polymerization. The degree of substitution of methoxyl groups was 1.80 ± 0.03.

Physical Properties

As the sedimentation coefficient decreased markedly with increasing concentration, s° was determined from a plot of reciprocal sedimentation coefficient *vs.* concentration (Fig. 1), and the results are given in Table II. Generally, s° increases with DP. However, s° of MC showed little DP dependence and rather decreased with increasing DP. It is known that gel formation of a solution decreases the apparent sedimentation coefficient. It was possible that interaction between MC molecules remained even at low concentration under the measurement conditions.

High-speed sedimentation equilibrium measurement was performed to study the intermolecular interaction of MC molecules in solution. As an example, a plot of concentration against the squared distance from the rotational center (r^2) for 0.1% (w/v) MC4000 is shown

Table I. Substitution Positions and Content (%) of Methoxyl Groups in MCs

Sample	Substitution positions							
	2,3,6	2,6	3,6	2,3	2	3	6	Non
MC25	20.0	31.7	7.0	6.2	15.1	2.1	12.4	5.6
MC50	20.2	28.2	7.7	5.1	16.7	4.3	11.5	6.3
MC400	22.2	25.6	7.5	9.2	15.7	2.9	12.4	4.4
MC1500	19.5	26.2	11.3	10.3	14.3	3.8	10.9	3.8
MC4000	20.6	26.6	10.9	7.2	14.4	4.1	11.6	4.5

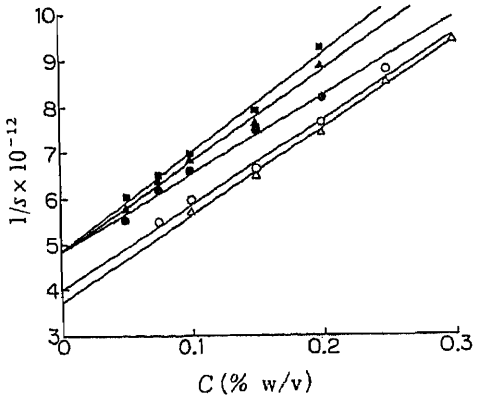

Fig. 1. Variation of Reciprocal Sedimentation Coefficient ($1/s$) with Concentration (C) for MCs

A 0.05 M KBr solution was used as the solvent. All runs were carried out at 60000 rpm at 20 °C. (△), MC25; (○), MC50; (●), MC400; (▲), MC1500; (■), MC4000.

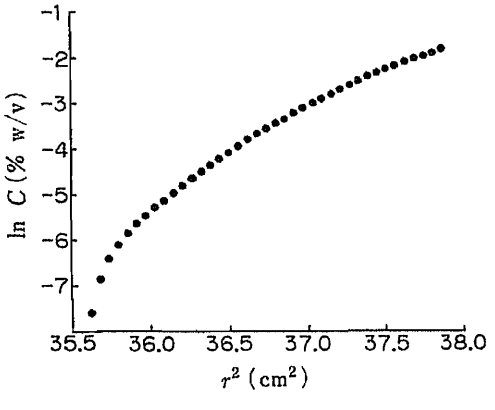

Fig. 2. Variation of Concentration (C) with Squared Distance from the Rotating Center (r^2) at Sedimentation Equilibrium

Results are for 0.1% (w/v) MC4000 in 0.05 M KBr solution at 13310 rpm at 20 °C.

in Fig. 2. If the sample is homogeneous and an ideal solution, a straight line should be obtained in this plot. Since the MC sample was polydisperse, it was expected that this plot would show a downward curvature. Each MC solution measured, however, showed a convex-upward curve (Fig. 2), suggesting that MC solutions have a large nonideality under these measurement conditions. The apparent molecular weight (M_{app}) was calculated on each point of this curve, and from the linear region of the $1/M_{app}$ *vs.* concentration plot the second virial coefficient (B) was obtained. The results are listed in Table II.

A change in B value in terms of intermolecular interaction of MC molecules in dilute solution was seen at about DP 500. Further, in each group of more than DP 500 (high DP) and of less than this (low DP), intermolecular interaction was similar and also independent of DP. These results for high DP samples may reflect properties of the whole solution rather than properties of isolated individual molecules, because of the large interaction between MC molecules under the solution conditions employed.

Viscosity of Dilute Solution

As shown in Fig. 3, for five MC samples the apparent concentration dependence of reduced viscosity increased with increasing DP. For MC aqueous solution and CMC in 0.15 M NaCl solution, the value of intrinsic viscosity, and k_1' and k_2' are given in Table II. A convenient index of the size (hydrodynamic volume) of isolated polymer coils is intrinsic viscosity. These values of both polysaccharides increased with increasing DP. The intrinsic viscosity of MC was only about one-half that of CMC of similar DP. The reasons for this may

TABLE II. Physical Properties of MCs and CMCs

Sample	DP	s°	$B \times 10^{-5}$	$[\eta] \times 10^{-3}$ Huggins constants		
				(l/residue)	k_1'	k_2'
MC25	330	2.70	8.13	3.62	0.268	0.154
MC50	440	2.51	8.08	4.88	0.176	0.172
MC400	710	2.08	3.48	8.24	0.270	0.198
MC1500	850	2.09	2.98	9.37	0.385	0.169
MC4000	1020	2.07	3.34	11.64	0.378	0.248
CMC500	500	—	—	12.27	0.154	0.085
CMC1050	1050	—	—	24.70	0.114	0.261

For dilute solution viscometry, aqueous solution (for MC) and 0.15 M NaCl solution (for CMC) were used.

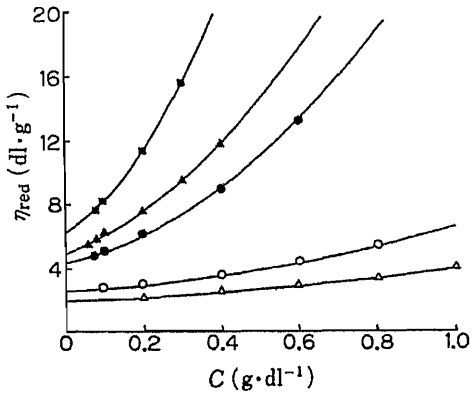

Fig. 3. Variation of Reduced Viscosity (η_{red}) with Concentration (C) for MCs

Symbols are as in Fig. 1.

TABLE III. Dilute Solution Viscometry Data of MCs in 50% Ethanol

Sample	$[\eta] \times 10^{-3}$	Huggins constants	
	(l/residue)	k_1'	k_2'
MC25	3.97	0.262	0.126
MC50	4.91	0.303	0.130
MC400	7.94	0.358	0.168
MC1500	10.38	0.261	0.199
MC4000	12.89	0.342	0.205

be as follows: a) CMC molecules even in the presence of NaCl may form extended coils owing to mutual repulsion between the negatively charged carboxyl groups; b) MC molecules may form compact coils owing to hydrophobic interaction between methoxyl groups; c) both factors may be important.

If MC molecules were extended coils in aqueous solution, it was expected that they would form compact coils on adding ethanol because of the reduction in affinity between MC molecules and the solvent. The results of MC samples in 50% ethanol were similar to those in aqueous solution (Tables II and III). This could be interpreted by assuming that since about half the hydroxyl groups of MC are substituted with methoxyl groups, MC molecules may form relatively compact coils by hydrogen bonding between free hydroxyl groups.

The constant k_1' is believed to be determined by the kind of polymer population and the solvent. This value of MC was higher than that of CMC of similar DP (Table II), suggesting that the intermolecular interaction of the MC sample was greater than that of the CMC sample at low concentration. This may be interpreted in terms of mutual repulsion for CMC molecules and hydrophobic interaction for MC molecules. It was noted that the k_1' value of high DP samples were larger than those of low DP samples even with the same type of polymer. In the case of k_2', which shows concentration dependence of higher order, DP dependence was observed for both polymers. This is presumably a result of greater ease of physical entanglement between polymer chains in relatively concentrated solutions.

An approximately linear relationship existed between DP determined from sedimentation equilibrium analysis and intrinsic viscosity in dilute aqueous solution.

$$[\eta] = 1.12 \times 10^{-5} \mathrm{DP}$$

This equation had 2.2% root-mean-square deviation from the observed values.

Concentration Dependence of "Zero-Shear" Viscosity

Figure 4 shows the shear rate dependence of apparent viscosity for various concentrations of MC1500 solutions. Similar results were obtained for other high DP sample solutions. For dilute solutions the reduction in viscosity from the maximum value with increasing shear rate was relatively small, these solutions being close to Newtonian fluids. For concentrated solutions the shear-thinning was much more dramatic. This non-Newtonian viscosity is said to be attributable to orientation of polymer chains in the direction of flow. With increase in concentration, shear-thinning was shifted to lower shear rate. This may result from the development of a dynamic entangled network structure between polymer coils in solution,[8] with a relaxation time similar to the time scale of measurement. For low DP samples, the shear rate dependence was relatively small even in concentrated solution. The decrease in viscosity was observed only at the high shear rate region (above $200\,\mathrm{s}^{-1}$).

Figure 5 shows the concentration dependence of "zero-shear" specific viscosity (η_{sp}) for five MC samples. In this case, double logarithmic plots of η_{sp} against concentration showed a

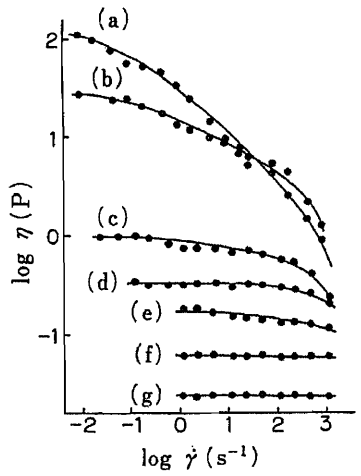

Fig. 4. Variation of Apparent Viscosity (η) with Concentration and Shear Rate (γ) for MC1500

(a), 3.0% (w/v); (b), 2.0% (w/v); (c), 1.0% (w/v); (d), 0.8% (w/v); (e), 0.6% (w/v); (f), 0.4% (w/v); (g), 0.2% (w/v).

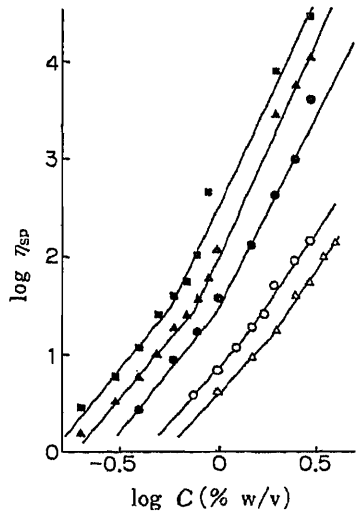

Fig. 5. Variation of "Zero-Shear" Specific Viscosity (η_{sp}) with Concentration (C) for MCs

Symbols are as in Fig. 1.

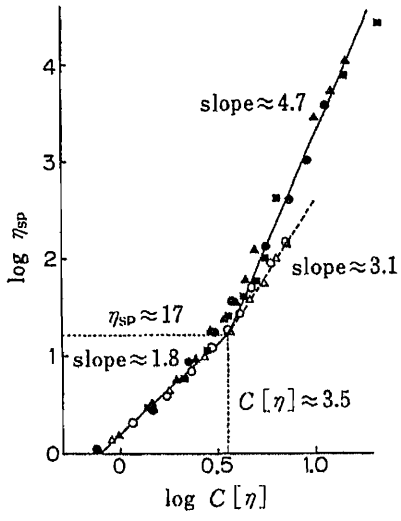

Fig. 6. Variation of "Zero-Shear" Specific Viscosity (η_{sp}) with Coil Overlap Parameter ($C[\eta]$) for MCs

Symbols are as in Fig. 1.

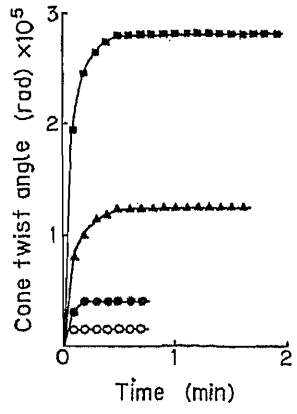

Fig. 7. Time Course of Cone Twist Angle for 3.0% w/v) MCs at Steady Shear Rate

Shear rate was $7.48 \times 10^{-3}\,s^{-1}$. The value for MC25 solution was not measurable. Symbols are as in Fig. 1.

pronounced increase in slope above a specific critical concentration (C^*). Similar behavior is well known for synthetic polymer solutions,[1] and is attributed to the transition from dilute solution conditions, where individual polymer molecules are present as isolated coils, to concentrated solutions where the total hydrodynamic volume of chains exceeds the volume of solution. C^* marks the onset of coil overlap and interpenetration.[9]

For random coil polymer solutions, $[\eta]$ varies with coil dimensions according to the Flory–Fox relationship:

$$[\eta]=\Phi 6^{3/2}R^3/M_r$$

where R is the radius of gyration, M_r is molecular weight, and Φ is a constant.[10] If treated as a sphere of radius R, hydrodynamic volume is proportional to $[\eta]M_r$. Since the total volume of coils is proportional to C/M_r, the degree of occupancy of coils may be characterized by the coil overlap parameter, $C[\eta]$. As shown in Fig. 6, the C^* transition occurs at a value of $C[\eta]$ close to 3.5 (*i.e.*, $C^*=3.5/[\eta]$), and the η_{sp} at this degree of coil overlap approaches 17. Since the viscosity of water at 21 °C is 0.982×10^{-2} poise, this corresponds to a solution viscosity of 0.17 P.

At a low degree of coil overlap (dilute solution), the slope was close to 1.8 for all MC samples, whereas at high degree of overlap (concentrated solutions) it was separated into two lines, low and high DP samples, with slopes of close to 3.1 and 4.7, respectively. These results are in good agreement with data on many linear polysaccharides[1] except that the slope of 4.7 for high DP samples is higher than that found for purely physical entanglement interaction (3—4), being close to that observed for Guar Gum (5.1).[10] This behavior may be attributed to the occurrence, in addition to normal physical entanglement, of specific interaction ("hyper-entanglement") between polymer chains.

A plot of the cone twist angle against time with a constantly rotating plate (shear rate: $7.48\times10^{-3}\,s^{-1}$) showed a relaxational phenomenon (Fig. 7). In the case of high DP samples, the time required for constancy of the cone twist angle, *i.e.*, the time required for steady-state flow to develop, was increased with increasing DP. This suggested the development of a dynamic network structure based on intermolecular interaction.

Dynamic Viscoelasticity

Dynamic viscosity (η'), complex modulus (G^*) and loss angle (θ) were calculated from G' and G'' by using the equations,

$$\eta'=G''/\omega$$

$$G^*=[(G')^2+(G'')^2]^{0.5}$$

$$\theta=\tan^{-1}(G''/G')$$

where ω is angular frequency. The angular frequency dependence of dynamic viscosity was similar to the shear rate dependence of steady-state viscosity for concentrated solutions. Dynamic viscosity was decreased with increasing angular frequency, and the curves were

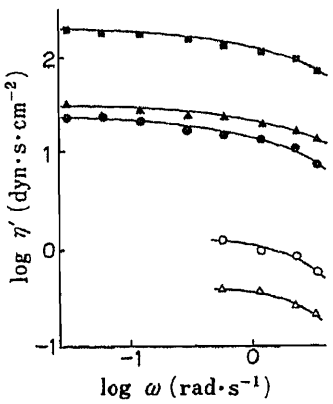

Fig. 8. Angular Frequency (ω) Dependence of Dynamic Viscosity (η') for 3.0% (w/v) MCs

Symbols are as in Fig. 1.

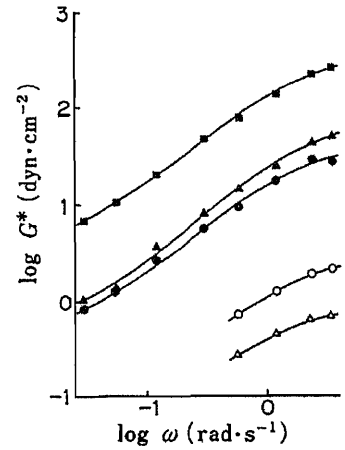

Fig. 9. Angular Frequency (ω) Dependence of Complex Moduli (G^*) for 3.0% (w/v) MCs

Symbols are as in Fig. 1.

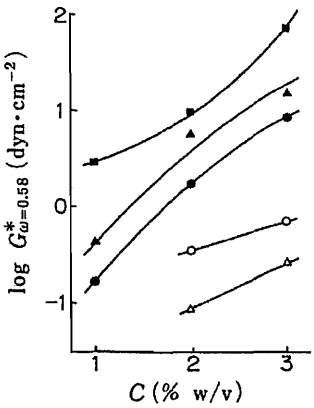

Fig. 10. Concentration (C) Dependence of Complex Moduli (G^*) at $\omega=0.58$ rad·s^{-1} for MCs

Symbols are as in Fig. 1.

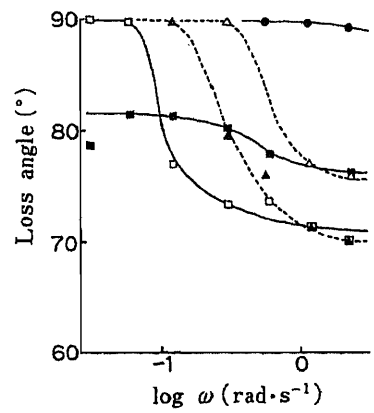

Fig. 11. Angular Frequency (ω) Dependence of Loss Angle

(■), 3.0% (w/v) MC4000; (□), 2.0% (w/v) MC4000; (▲), 2.0% (w/v) MC1500; (△), 1.0% (w/v) MC1500; (●), 3.0% (w/v) MC400.

parallel for various values of DP (Fig. 8).

Complex moduli for concentrated MC solutions were increased with increasing angular frequency in the frequency range measured (Fig. 9). Again the curves were parallel for various values of DP, suggesting that the dynamic viscoelastic behavior of MC solution at a given concentration greatly depended on DP. Figure 10 shows a plot of G^* at $\omega=0.58$ rad·s^{-1} against concentration. With increase in DP, the concentration dependence was increased over the concentration range measured. This dependence of high DP samples was different from that of low DP samples. For low DP sample solutions at 1.0% (w/v), the dynamic viscoelasticity was too small to measure.

As shown in Fig. 11, the loss angle of low DP samples was close to 90° even in concentrated solution throughout the frequency range measured, *i.e.*, these solutions behaved as viscous liquids. In contrast, high DP sample solutions showed a remarkable change in loss angle with increasing angular frequency, particularly in the high frequency region. This dependence appeared to be in the order of 3.0% (w/v) MC4000, 2.0% (w/v) MS 4000, 2.0% (w/v) MC1500, 1.0% (w/v) MC1500, 3.0% (w/v) MC400. The angular frequency dependence of loss angle may change continuously and be related to both DP and concentration. These results suggest the formation of a dynamic network structure between MC molecules of high DP samples in solution.

For MC400, in spite of the high DP, the angular frequency dependence of loss angle for 1.0% (w/v) solution was similar to that of low DP sample solutions, and the solution behaved as a viscous liquid. Presumably because the 1.0% (w/v) concentration of this sample is close to the critical concentration and there was no hyperentanglement, differing from other high DP sample solutions, no dynamic network structure developed.

Conclusion

MC molecules in solution are considered to be compact coils. When they are present individually in dilute solution, a hydrophobic environment is formed within each coil, allowing relatively compact coils. As the concentration of MC is increased, each molecule is brought into contact with others. This may be accompanied with a change in MC molecular form, due to interactions such as coil overlapping, hydrogen bonding and/or hydrophobic bonding, leading to a dramatic increase in the concentration dependence of their rheological

properties. The intermolecular interaction of MC samples could be divided into two groups depending on their DPs. This suggests that the increase in DP caused an increase not only in molecular size but also in intermolecular interaction of MC molecules in solution. In particular, interesting rheological properties of MC solutions were observed for MCs with DP above 500, when the concentration was higher than a critical concentration. The viscoelastic behavior of high DP samples in concentrated solution depended significantly on both DP and concentration.

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Inactivation of Lysozyme in a Solution Containing Sodium Bisulfite under Scattered Light

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When a solution (pH 4.0) containing 1 mg/ml hen egg-white lysozyme (EC 3.2.1.17) and 1 mM sodium bisulfite (SBS) was stored at 3 °C under scattered light, the residual activity of lysozyme was reduced to about 10% within 3 d. The inactivation was dependent on the pH of the medium, and was especially marked at pH below 5. It was largely prevented by shielding the solution from light, by replacement of the dissolved oxygen in the medium by nitrogen gas and by the addition of several radical scavengers to the medium prior to storage. The absorbance of lysozyme treated with SBS for 3 d under scattered light was decreased by about 10% at 280 nm, and the content of tryptophan residues of lysozyme was decreased by about 1.0 mol of Trp/mol of enzyme. The change in the state of aromatic amino acid residues of lysozyme treated with SBS under scattered light was observed by circular dichroism spectral analysis in the wavelength range of 250—340 nm, and this change was prevented by shielding from light and by addition of radical scavengers.

These results suggest that the inactivation of lysozyme during storage in a solution containing SBS was due to modification or destruction of amino acid residues in lysozyme by free radicals formed during the autooxidation of bisulfite under scattered light.

Keywords——lysozyme; sodium bisulfite; scattered light; lysozyme inactivation; storage solution; free radical; aromatic amino acid; tryptophan

It is well known that various drugs, such as ascorbic acid, cyanocobalamin, penicillins and cephalosporin C's are degraded by sodium bisulfite (SBS),[1] which is frequently added to drug preparations as an antioxidant. Previously, we reported that in a syrup preparation of lysozyme chloride mixed with a syrup preparation of trimetoquinol hydrochloride and stored for a few days under scattered light, lysozyme was inactivated light-intensity-dependently, and we suggested that the inactivation might be due to (bi)sulfite contained in the syrup preparation of trimetoquinol hydrochloride as an antioxidant.[2] On the other hand, studies on the autooxidation of (bi)sulfite have shown that the reaction mechanism involves free radicals.[3] Free radicals formed during autooxidation of bisulfite inactivate certain enzymes, such as papain, through modification of amino acid residues in the active site.[4]

In this study, we investigated the mechanism of inactivation of lysozyme during storage in a solution containing SBS under scattered light.

Materials and Methods

Materials——Hen egg-white lysozyme and dehydrated cells of *Micrococcus lysodeikticus* were purchased from Sigma Chemicals Co., Ltd., St. Louis, MO. Sodium bisulfite, cysteine hydrochloride, dimethyl sulfoxide (DMSO), urea, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-bromosuccinimide (NBS) were purchased from Nakarai Chemicals Co., Ltd., Kyoto, Japan. All other chemicals were of the highest purity available, and used without further purification.

Samples and Storage Conditions——Unless otherwise specified, samples for storage were solutions containing 1 mg/ml lysozyme, 1 mM sodium bisulfite and 100 mM citrate buffer (pH 4.0) in a final volume of 10 ml. The

concentrations of lysozyme and sodium bisulfite, and the pH of the medium were in the range of clinical use. These solutions were stored in polypropylene bottles for 3—7 d at 3 °C under scattered light (550 lux) or in the dark. The inner volume of the polypropylene bottle used was 45 ml, and the volume ratio of the sample solution to air in the bottle was 0.29. A 40 W fluorescent lamp (white light, type FLR SW/M, Matsushita Denki Co., Ltd., Osaka, Japan) was used as the light source.

Enzyme Assay——Lysozyme was assayed by the method of Azari with *M. lysodeikticus* cells as a substrate.[5] The change in turbidity of the cell suspension was recorded at 450 nm with a double-beam recording spectrophotometer (Shimadzu, model UV-300).

Determination of Sulfhydryl Groups of Lysozyme——After removal of the SBS from the stored sample by passage through a Sephadex G-25 column equilibrated with 100 mM acetic acid buffer (pH 4.0), the sulfhydryl groups of lysozyme were determined in the presence of 8 M urea by the method of Ellman with DTNB.[6]

Determination of Tryptophan Residues in Lysozyme——After removal of the SBS from the stored sample by dialysis overnight against 6 liters of 100 mM acetate buffer (pH 4.5) at 3 °C, tryptophan residues were determined by the method of Spande and Witkop[7] in the presence of 8 M urea. Lysozyme concentration was corrected for the change of volume of the solution after dialysis.

Measurement of the Circular Dichroism (CD) Spectra of Lysozyme——Measurement of CD spectra was carried out using a spectropolarimeter (Nihon Bunko Kogyo, Co., Ltd., model J-500 A) equipped with a 1.0 cm cell holder maintained thermostatically at 25 °C.

Results

Inactivation of Lysozyme by SBS under Scattered Light

Figure 1 shows the change in lysozyme activity in a solution stored at 3 °C under scattered light or in the dark. Lysozyme was inactivated by SBS dose dependently under scattered light, and after storage for 2 d, the residual activities at the concentrations of 0.6 and 1.0 mM SBS were about 10% of the control activity. On the other hand, no significant inactivation of lysozyme was observed after storage in the dark for at least 3 d.

To examine whether the inactivation of lysozyme occurring under these storage conditions was reversible or not, lysozyme activity was measured after removal of the SBS from the stored sample on a Sephadex G-25 column. Lysozyme activity, however, was not restored at all (data not shown).

As shown in Fig. 1, the lysozyme activity in the solution stored under scattered light was reduced dramatically within 3 d. Therefore, unless otherwise specified, a 3-d storage period was used in the following experiments.

Figure 2 shows the effect of the pH of the medium on the inactivation of lysozyme. In the dark, the lysozyme was not inactivated in the pH range of 4 to 10, but under scattered light, it was inactivated depending on the pH, especially at pH below 5.

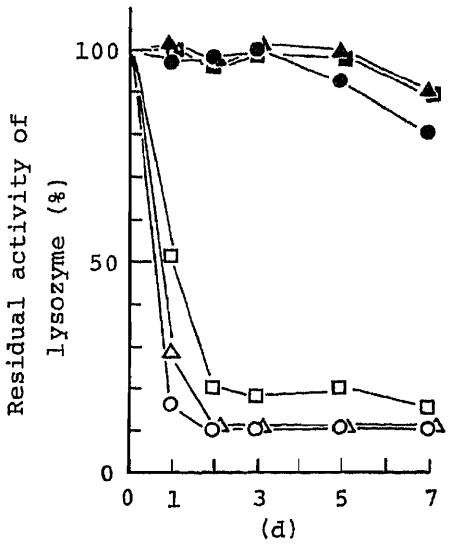

Fig. 1. Inactivation of Lysozyme in a Solution Containing SBS under Scattered Light

Solutions (pH 4.0) containing 1 mg/ml lysozyme and 1.0 (○), 0.6 (△) or 0.4 (□) mM SBS were stored at 3 °C under scattered light (550 lux), and lysozyme activity was measured daily for 7 d. Another experiment was run in the dark (closed symbols).

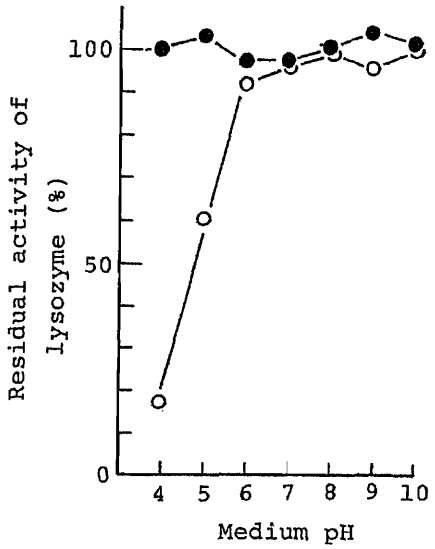

Fig. 2. Effect of pH on the Inactivation of Lysozyme by SBS

Solutions containing 1 mg/ml lysozyme and 1 mM SBS in 100 mM citrate buffer (pH 4 and 5), 100 mM phosphate buffer (pH 6, 7 and 8) or 100 mM carbonate buffer (pH 9 and 10) were stored at 3 °C for 1 d in 550 lux of scattered light (○) or in the dark (●).

Table I. Inhibitory Effect of SBS on Lysozyme Activity under Anaerobic Conditions

Conditions		Residual activity of lysozyme (%)
$-NaHSO_3$	in air	100
	in N_2 gas	100
$+NaHSO_3$	in air	14
	in N_2 gas	63

The oxygen dissolved in the solution (pH 4.0) containing 1 mg/ml lysozyme with or without 1 mM SBS was replaced by nitrogen gas. The solution was then stored for 3 d at 3 °C under scattered light (550 lux).

Table II. Effect of Radical Scavengers on the Inactivation of Lysozyme in a Solution Containing SBS under Scattered Light

Substance added	Concentration (mM)	Residual activity (%)
None	—	100
$NaHSO_3$	1	14
$NaHSO_3$ + KI[b]	10	102
	1	89
	0.1	25
$NaHSO_3$ + cysteine[b]	10	100
	1	102
	0.1	104
$NaHSO_3$ + KBr[b]	10	86
	1	38
	0.1	33
$NaHSO_3$ + ethanol[a]	10	43
$NaHSO_3$ + DMSO[a]	10	47
$NaHSO_3$ + D-mannitol[a]	100	48

The solution (pH 4.0) contained 1 mg/ml lysozyme, 1 mM SBS and the specified amount of various radical scavengers. The solution was stored for 1 (*a*) or 3 (*b*) d at 3 °C under scattered light (550 lux).

Table I shows the change in lysozyme activity in the solution after the dissolved oxygen had been replaced by nitrogen gas. The inactivation of the lysozyme was largely prevented. These results suggest the participation of oxygen in the inactivation of the lysozyme by SBS.

Table II shows the change in the lysozyme activity in the stored solution when various radical scavengers such as KI, cysteine, ethanol, DMSO, D-mannitol and KBr were added. All of them significantly prevented the inactivation of lysozyme.

Determination of Tryptophan Residue in Lysozyme Stored in a Solution Containing SBS under Scattered Light

Figure 3 shows ultraviolet (UV) spectra of lysozyme stored for 3 d in a solution containing SBS under scattered light. The absorbance at 280 nm due to aromatic amino acid residues was decreased by about 10% as compared with intact lysozyme. To observe the effect of SBS on tryptophan residues in lysozyme, the number of tryptophan residues was determined. As shown in Table III, the lysozyme stored in a solution containing SBS for 3 d in the dark, which retained 100% activity, had the same number of tryptophan residues (5.78 mol of Trp/mol of enzyme) as the control (5.89 mol of Trp/mol of enzyme). On the other hand, the lysozyme stored in the solution containing SBS under scattered light for 3 d had 4.78 mol of Trp/mol of enzyme. These results suggest some destruction or modification of tryptophan residues in the lysozyme treated with SBS under scattered light.

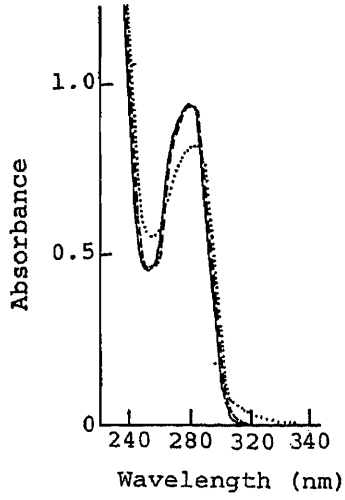

Fig. 3. UV Spectra of Lysozyme Stored in a Solution Containing SBS under Scattered Light or in the Dark

Solutions (pH 4.0) containing 1 mg/ml lysozyme and 1 mM SBS were stored for 3 d in 550 lux of scattered light (------) or in the dark (----). The full line shows the spectrum of intact lysozyme.

TABLE III. Number of Tryptophan Residues in Lysozyme Stored in a Solution Containing SBS under Scattered Light

Sample		Tryptophan residues (mol/mol of enzyme)
Untreated (control)		5.89
$NaHSO_3$ treated	in the dark	5.87
	in 550 lux	4.78

The solution (pH 4.0) containing 1 mg/ml lysozyme and 1 mM SBS was stored for 3 d at 3 °C under scattered light (550 lux) or in the dark.

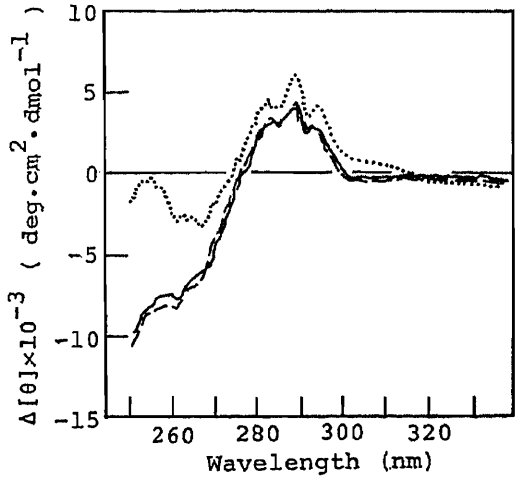

Fig. 4. CD Spectra of Lysozyme Stored in a Solution Containing SBS under Scattered Light or in the Dark

Solutions (pH 4.0) containing 1 mg/ml lysozyme and 1 mM SBS were stored for 3 d in 550 lux of scattered light (------) or in the dark (----). The full line shows the spectrum of intact lysozyme.

CD Spectra of Lysozyme Stored in a Solution Containing SBS under Scattered Light

To investigate the change in the state of aromatic amino acid residues in lysozyme in the stored solution, the CD spectrum of the lysozyme was measured in the wavelength range of 250—340 nm. As shown in Fig. 4, the CD spectrum of the lysozyme inactivated by the storage under scattered light was enhanced in the ranges of 250—265 nm and 275—300 nm in comparison with that of intact lysozyme. On the other hand, no change occurred in the CD spectrum of lysozyme stored in the dark, and the lysozyme was not inactivated. These results indicate that the inactivation of lysozyme by SBS under scattered light is due to a change in aromatic amino acid. In the case of the solution containing 10 mM KI as a radical scavenger, the lysozyme was not inactivated and the CD spectrum was not changed (data not shown).

Determination of Sulfhydryl Groups in Lysozyme Stored in a Solution Containing SBS under Scattered Light

To examine whether scissions of the disulfide bonds in lysozyme had occurred or not, sulfhydryl groups were determined, but they were not detected in the inactivated lysozyme (data not shown).

Discussion

The presence of dissolved oxygen and the pH of the medium were found to be important factors in relation to the inactivation of lysozyme in the presence of SBS under scattered light. Bisulfite dissociates according to the following reaction;

$$HSO_3^- + H_2O \rightleftharpoons H_3O^+ + SO_3^{2-}$$

It is well known that the equilibrium between bisulfite and sulfite depends upon the pH of the medium and the pK_a of the dissociation of bisulfite to sulfite is 6.25—7.20.[9] Since lysozyme activity was markedly reduced at pH below 5 (Fig. 2), the reactive species is considered to be mainly bisulfite.

The existence of a free radical chain mechanism for the aerobic autooxidation of (bi) sulfite to (bi) sulfate has been well documented,[3] and light and heat have been reported to be regulating factors of this chain reaction.[3,10] Free radicals such as $\cdot O_2^-$, $\cdot OH$ and $\cdot SO_3H$ are generated during the aerobic oxidation of bisulfite and these radicals in turn propagate the bisulfite–oxygen chain reaction.[3] An anaerobic condition or $\cdot OH$ and $\cdot O_2^-$ scavengers prevented the inactivation of lysozyme in the presence of bisulfite under scattered light (Tables I and II). Consequently, it is suggested that free radicals formed during autooxidation of bisulfite under scattered light take part in the inactivation of lysozyme. It has been reported that the oxidizing radicals formed during autooxidation of bisulfite can induce the oxidation and destruction of a number of amino acids, such as methionine[11] and tryptophan.[12] Free radicals produced during the aerobic oxidation of bisulfite have been reported to inactivate papain through modification of amino acid residues in the active site.[4] Thus, the mechanism of inactivation of lysozyme by free radicals may be the modification or destruction of amino acid residues which play an important role in the appearance of lysozyme activity. This was supported by the reduction of absorbance at 280 nm and the enhancement of the CD spectrum in the ranges of 250—265 nm and of 275—300 nm in lysozyme treated with bisulfite under scattered light. Furthermore, about 1.0 mol of the tryptophan residue per mol of enzyme was shown to be lost (Table III). These findings indicate that the inactivation of lysozyme was due to the destruction or modification of tryptophan by the free radicals formed during the autooxidation of bisulfite under scattered light.

On the other hand, as described in a previous paper,[2] another possible mechanism of the inactivation is the scission of disulfide bonds by sulfite ions[13];

$$RSSR + SO_3^{2-} \longrightarrow RS^- + RSSO_3^-$$
$$\uparrow \quad \downarrow$$
$$[O]$$

Sulfitolysis has frequently been used for the cleavage of disulfide bonds in proteins, usually in the presence of a protein-denaturant and/or an oxidizing agent. In the present case, it appears that sulfitolysis of disulfide bonds in the protein by sulfite is not the main reaction of inactivation. This was deduced from the following findings and facts. Radical scavengers, such as cysteine and KI, prevented the inactivation (Table II) and the change of CD spectrum observed in the presence of SBS under scattered light (Fig. 4); no appreciable cleavage of disulfide bonds was found from the determination of sulfhydryl groups by DTNB; disulfide bonds in lysozyme are resistant to reducing agents, and are susceptible to reduction only in the presence of concentrated protein-denaturant, such as urea[14]; if all RSSR turn to $RSSO_3^-$, they would not react with DTNB, but it is considered that the formation of $RSSO_3^-$ does not occur, since SH is scarcely oxidized under acidic conditions (the medium pH was 4.0); it has been shown that the reactive species participating in the scission of disulfide is SO_3^{2-} and the optimal conditions of sulfitolysis are neutral-alkaline[14]; lysozyme was strongly inactivated under acidic conditions (Fig. 2). Therefore it seems that sulfitolysis is not involved in the inactivation of lysozyme.

In conclusion, it is suggested that inactivation of lysozyme during storage in a solution containing SBS under scattered light was due to modification or destruction of a tryptophan residue of lysozyme by free radicals formed during the autooxidation of bisulfite.

Acknowledgements The authors are indebted to Dr. J. Yano and Miss S. Nishigaki, Nippon Shinyaku Co., Ltd., for measurement of CD spectra and for valuable discussions.

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Preparations and Reactions of (*Z*)-3-Arylidene-6-arylmethyl-2,5-piperazinediones Having Highly Oxygenated Benzene Rings

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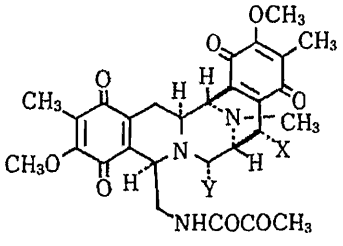
A simple and efficient synthesis of a variety of (*Z*)-3-arylidene-6-arylmethyl-2,5-piperazinediones **10** having highly oxygenated benzene rings, and regioselective benzylation at position 4 are described.

Keywords——dimeric isoquinolinequinone; saframycin; (*Z*)-3-arylidene-6-arylmethyl-2,5-piperazinedione; regioselective benzylation; pyrazine

In connection with our studies directed toward the synthesis of dimeric isoquinolinequinone antibiotics, *i.e.*, saframycins (**1a**—**c**),[1] safracins,[2] and renieramycins,[3] we considered the use of 3,6-bis(arylmethyl)-2,5-piperazinediones **2** to construct the 1,5-imino-3-benzazocine skeleton **3** of these antibiotics (Fig. 1).

The great majority of known substituted 2,5-piperazinediones are derived from natural amino acids.[4] However, the 3,6-bis(arylmethyl)-2,5-piperazinediones, possessing highly oxygenated benzene rings, have not been investigated except for a few limited cases.[5] On the other hand, (*Z*)-3-arylidene-2,5-piperazinediones have been utilized effectively in the synthesis of 3-arylmethyl-2,5-piperazinediones because of their stereoselective hydrogenation.[6] In this report, a simple and efficient synthesis of (*Z*)-3-arylidene-6-arylmethyl-2,5-piperazinediones **10a**—**f** and regioselective benzylation of **10a** to a mono-*N*-benzyl-2,5-piperazinedione **11**, are described.

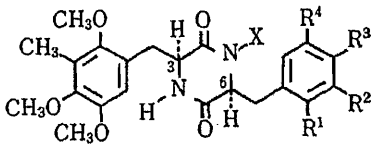
A mixture of 2,4,5-trimethoxy-3-methylbenzaldehyde (**4a**)[7] and 1,4-diacetyl-2,5-piperazinedione (**5**)[8] in dimethylformamide (DMF) was treated with potassium *tert*-butoxide in *tert*-butyl alcohol at room temperature for 24 h to afford 3-arylidene-2,5-piperazinedione **6** in 66% yield according to Gallina and Liberatori.[9] In the proton nuclear magnetic resonance



1a : X=H, Y=CN

1b : X=Y=H

1c : X=OCH_3, Y=H



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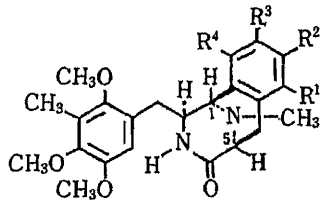

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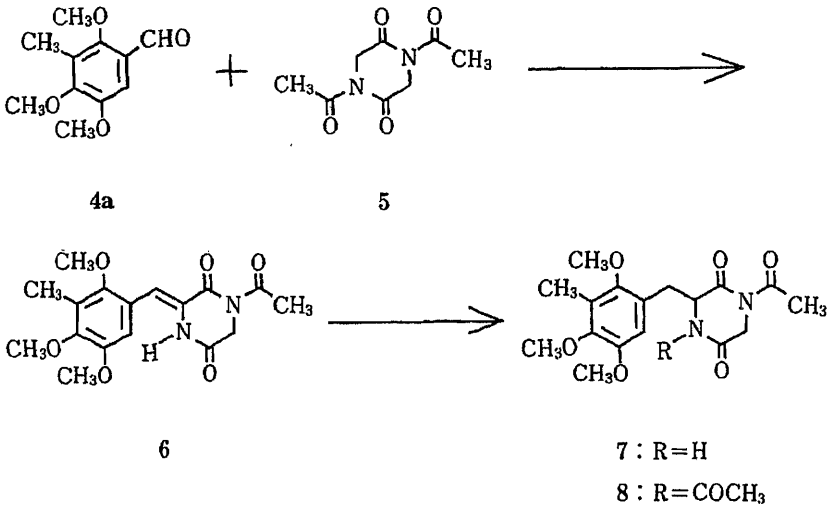
Fig. 1



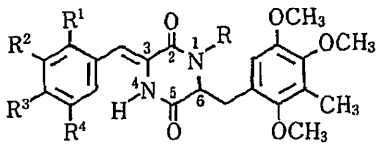
4a 5

6

7 : R=H

8 : R=COCH$_3$

Chart 1



9 : R=COCH$_3$

10 : R=H

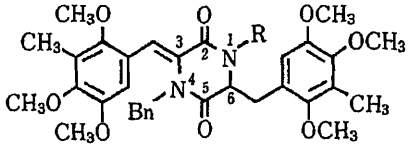
	R^1	R^2	R^3	R^4
a	OCH$_3$	CH$_3$	OCH$_3$	OCH$_3$
b	OBn	CH$_3$	OCH$_3$	OCH$_3$
c	OCH$_3$	CH$_3$	OCH$_3$	OBn
d	H	OCH$_3$	CH$_3$	H
e	OCH$_3$	OCH$_3$	CH$_3$	OCH$_3$
f	OBn	OCH$_3$	CH$_3$	OCH$_3$

Fig. 2

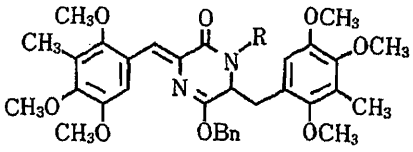
(^{1}H-NMR) spectrum of **6**, the signal due to the olefinic proton at δ 7.03 (1H, s) indicated that the geometry of the arylidene was *Z*, and the *E* isomer could not be detected.[10] Catalytic reduction of **6** in ethanol and DMF in the presence of 10% palladium on carbon gave **7**, which upon treatment with acetic anhydride at 110 °C for 4 h afforded the 1,4-diacetyl derivative **8** in 80% yield (Chart 1).

The highly substituted benzaldehydes **4a—f** were condensed with **8** under the same conditions as used for the reaction of **4a** with **5** to give the desired 3-arylidene-6-arylmethyl-2,5-piperazinediones **9a—f** in 58—84% yields. Furthermore, treatment of **9a—f** with hydrazine hydrate in DMF at room temperature afforded the deacylated compounds **10a—f** in 51—89% yields. The structures of **9a—f** and **10a—f** were assigned on the basis of elemental analysis, infrared (IR), mass (MS), ultraviolet (UV), and ^{1}H-NMR spectra. However, the geometries of **9a—f** and **10a—f** were not defined by their ^{1}H-NMR spectra since the olefinic proton and aromatic proton signals could not be assigned. Porter and Sammes[11] reported that the *E* isomer of the 1-acetyl-3-benzylidene-2,5-piperazinedione could be acetylated by acetic anhydride at position 4, but the *Z* isomer did not give the acetylation product because of the steric hindrance of the bulky aromatic ring attached to the C-3 methylidene group. Thus, acetylation of **10a** with acetic anhydride at 110 °C for 4 h gave the 1-acetyl derivative **9a** in 71% yield. However, on similar treatment, **9a** failed to undergo further acetylation. These results indicated that **9a** and **10a** have *Z* configuration (Fig. 2).

Next, we planned to synthesize a mono-*N*-alkylated 2,5-piperazinedione at position 4. In general, alkylation of the 2,5-piperazinediones by lower alkyl halides gives a mixture from which mono-*N*-alkyl derivatives are not readily separated.[12] When the *Z* isomer **10a** was alkylated with benzyl bromide (1.1 eq) in the presence of sodium hydride (1.1 eq) in DMF at

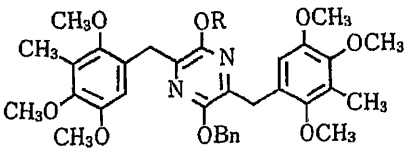


11 : R=H
13 : R=Bn
15 : R=$COCH_3$

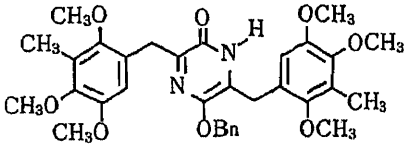


12 : R=H
14 : R=$COCH_3$

Fig. 3



16 : R=H
18 : R=Bn



17

Fig. 4

room temperature a mixture of several alkylation products was obtained. Separation of this mixture by column chromatography on silica gel afforded **11** (61%), **12** (8%), and **13** (22%). The structure of the main product **11** was determined on the basis of elemental analysis and spectral data. In particular, in the ^{1}H-NMR spectrum of **11**, a C-6 methine signal appeared at δ 4.38 (1H, m), while the corresponding signal of its acetate **15**, prepared quantitatively from **11** by treatment with acetic anhydride at 110 °C for 4 h, appeared at δ 5.50 (1H, t, J=7 Hz). The second product was the *O*-alkyl derivative **12**, which was treated with acetic anhydride to give the 1-acetyl compound **14**. The ^{1}H-NMR spectrum of **12** indicated a low-field shift (0.84 ppm) of the signal of the methine proton at position 6. This observation allowed us to make the regiochemical assignment for **12** as shown (Fig. 3). The third product, the 1,4-dibenzyl derivative **13**, was alternatively formed from **11** by treatment with benzyl bromide and sodium hydride in DMF in 70% yield. The selectivity observed in the formation of **11** might be mainly due to the enamine structure of **10a**, in which the amide proton at position 4 is more acidic than that at position 1.

Finally, treatment of **12** with methanolic potassium hydroxide afforded the corresponding pyrazine derivative **16**, whose ^{1}H-NMR spectrum indicated that only one compound was present in solution. Its IR spectrum showed a broad absorption between 3200—3000 cm^{-1} indicating that the major tautomeric form present was the pyrazine **16** rather than the pyrazinone **17**. On the other hand, its UV spectrum in methanol showed, besides the typical pyrazine absorption band at 324 nm, a band at 366 nm attributed to the pyrazinone **17**. The 2,5-dibenzyl ether **18** was produced by alkylation of **12** with benzyl bromide and sodium hydride in DMF in 67% yield. Its UV spectrum confirmed that long-wavelength absorption, assigned to the pyrazinone tautomer, was absent when the tautomerism was blocked by *O*-alkylation (Fig. 4).[13]

Thus, we succeeded in a simple and efficient synthesis of a variety of (*Z*)-3-arylidene-6-arylmethyl-2,5-piperazinediones **10a—f** having highly oxygenated benzene rings, and regioselective benzylation at position 4 of **10a**. An extension of this work and application of **11** to the total synthesis of saframycin B (**1b**) will be reported elsewhere.[14]

Experimental

All melting points were taken on a Yanagimoto micro-melting point apparatus and are uncorrected. Mass spectra were recorded on a Nippon-Denshi JMS-D 300 and IR spectra on a Hitachi 260-10 spectrophotometer. UV spectra were recorded with a Hitachi 340 spectrometer using methanol as the solvent. ^{1}H-NMR spectra were obtained on a Nippon-Denshi PS-100 (100 MHz) spectrometer, with tetramethylsilane (TMS) as an internal standard. ^{13}C-NMR spectra were determined with Nippon-Denshi GX 400 (100 MHz) spectrometers. Microanalytical data were obtained by using a Perkin-Elmer 240 elemental analyzer.

Materials——2,4,5-Trimethoxy-3-methylbenzaldehyde (**4a**, bp 132—134 °C/3 mmHg)[7] was prepared from *p*-benzoquinone in four steps [a) acetic anhydride, H_2SO_4, 50 °C; b) $(CH_3)_2SO_4$, NaOH; c) *n*-BuLi, $(CH_3)_2SO_4$, tetrahydrofuran (THF), room temperature[15]; d) $TiCl_4$, Cl_2CHOCH_3, CH_2Cl_2] in 55% yield. 1,4-Diacetyl-2,5-piperazinedione (**5**, mp 104.5—105 °C)[8] was prepared in 80% yield from glycine anhydride by treatment with acetic anhydride at 110—115 °C for 2 h. 2-Benzyloxy-4,5-dimethoxy-3-methylbenzaldehyde (**4b**, mp 62—63 °C) was prepared from 3,4-dimethoxy-2-methylphenol[16] in two steps [a) $TiCl_4$, Cl_2CHOCH_3, CH_2Cl_2; b) sodium hydride, benzyl bromide, DMF] in 53% yield. 2-Benzyloxy-3,5-dimethoxy-4-methylbenzaldehyde (**4f**, mp 70.5—71 °C) was prepared from 2-hydroxy-3,5-dimethoxy-4-methylbenzaldehyde[17] by treatment with sodium hydride and benzyl bromide in 87% yield.

(*Z*)-1-Acetyl-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-2,5-piperazinedione (6)——A solution of potassium *tert*-butoxide (11.22 g, 0.1 M) in *tert*-butyl alcohol (200 ml) was added to a stirred solution of the benzaldehyde **4a** (21 g, 0.1 M) and the diacetyl derivative **5** (19.8 g, 0.1 M) in dry DMF (200 ml). After being stirred for 24 h at room temperature, the reaction mixture was poured into water (600 ml) and extracted with AcOEt. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give the residue, recrystallization of which from AcOEt gave **6** (23.0 g, 66%) as pale yellow prisms, mp 173.5—175 °C. *Anal.* Calcd for $C_{17}H_{20}N_2O_6$: C, 58.61; H, 5.79; N, 8.04. Found: C, 58.58; H, 5.88; N, 7.82. MS *m/z* (%): 348 (M^+, 83), 275 (100). IR ν_{max}^{KBr} cm^{-1}: 3130, 1700, 1630. UV λ_{max} nm (log ε): 264 (3.75), 298 (3.98), 321 (4.03). ^{1}H-NMR ($CDCl_3$) δ: 2.23 (3H, s, $ArCH_3$), 2.64 (3H, s, $COCH_3$), 3.65 (3H, s, OCH_3), 3.86 (6H, s, 2 × OCH_3), 4.47 (2H, s, CH_2), 6.65 (1H, s, ArH), 7.03 (1H, s, C=CH), 9.27 (1H, s, NH).

1-Acetyl-3-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (7)——The arylidene derivative **6** (17.4 g, 50 mM) was dissolved in EtOH (50 ml) and DMF (50 ml) and hydrogenated over 10% palladium on carbon (2 g) at 1 atm for 4 h. After removal of the catalyst by filtration, the filtrate was evaporated and the residue was partitioned between AcOEt and water. The organic phase was dried over Na_2SO_4, and evaporated to give the residue, recrystallization of which from AcOEt gave **7** (17.0 g, 97%) as colorless prisms, mp 116—117.5 °C. *Anal.* Calcd for $C_{17}H_{22}N_2O_6$: C, 58.27; H, 6.33; N, 8.00. Found: C, 58.25; H, 6.42; N, 7.89. MS *m/z* (%): 350 (M^+, 6), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3370, 1715, 1685. UV λ_{max} nm (log ε): 282 (3.40). ^{1}H-NMR ($CDCl_3$) δ: 2.21 (3H, s, $ArCH_3$), 2.61 (3H, s, $COCH_3$), 3.06 (1H, dd, J=18, 7 Hz, ArCH), 3.30 (1H, dd, J=18, 5 Hz, ArCH), 3.71 (3H, s, OCH_3), 3.85 (6H, s, 2 × OCH_3), 4.03 (1H, d, J=18 Hz, NCH), 4.26 (1H, d, J=18 Hz, NCH), 4.35 (1H, dd, J=7, 5 Hz, CH), 6.58 (1H, s, ArH), 6.97 (1H, s, NH).

1,4-Diacetyl-3-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (8)——A solution of the monoacetyl derivative **7** (17.0 g, 48.6 mM) in acetic anhydride (200 ml) was heated at 110 °C for 4 h. Removal of the solvent *in vacuo* afforded the residue, which was partitioned between benzene and saturated $NaHCO_3$ solution. The organic phase was washed with water, dried over Na_2SO_4, and evaporated to give a solid. Recrystallization from benzene–Et_2O gave **8** (15.75 g, 83%) as colorless needles, mp 116.5—117 °C. *Anal.* Calcd for $C_{19}H_{24}N_2O_7$: C, 58.15; H, 6.17; N, 7.14. Found: C, 57.88; H, 6.19; N, 7.08. MS *m/z* (%): 392 (M^+, 16), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 1710. UV λ_{max} nm (log ε): 283 (3.58). ^{1}H-NMR ($CDCl_3$) δ: 2.13 (3H, s, $ArCH_3$), 2.57 (6H, s, 2 × $COCH_3$), 2.82 (1H, d, J=18 Hz, NCH), 3.16 (1H, dd, J=13, 4 Hz, ArCH), 3.32 (1H, dd, J=13, 6 Hz, ArCH), 3.59 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 4.53 (1H, d, J=18 Hz, NCH), 5.38 (1H, dd, J=6, 4 Hz, CH), 6.40 (1H, s, ArH).

(*Z*)-1-Acetyl-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-2,5-piperazinedione (9a)——A solution of potassium *tert*-butoxide (3.37 g, 30 mM) in *tert*-butyl alcohol (60 ml) was added to a stirred solution of the benzaldehyde **4a** (6.3 g, 30 mM) and the diacetyl derivative **8** (11.76 g, 30 mM) in dry DMF (60 ml). After being stirred for 24 h at room temperature, the reaction mixture was poured into water (200 ml), and extracted with benzene. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give the residue, recrystallization of which from benzene–Et_2O gave **9a** (13.2 g, 81%) as pale yellow prisms, mp 136—137.5 °C. *Anal.* Calcd for $C_{28}H_{34}N_2O_9$: C, 61.98; H, 6.32; N, 5.16. Found: C, 62.03; H, 6.38; N, 5.12. MS *m/z* (%): 542 (M^+, 22), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3220, 1700, 1635. UV λ_{max} nm (log ε): 230 (4.30), 256 sh (4.02), 295 (3.93), 335 (4.14). ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s, $ArCH_3$), 2.21 (3H, s, $ArCH_3$), 2.65 (3H, s, $COCH_3$), 2.94 (1H, dd, J=14, 2 Hz, ArCH), 3.26 (3H, s, OCH_3), 3.42 (1H, dd, J=6, 2 Hz, ArCH), 3.53 (3H, s, OCH_3), 3.57 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 4.01 (3H, s, OCH_3), 5.36 (1H, dd, J=6, 2 Hz, CH), 6.20 (1H, s), 6.31 (1H, s), 6.49 (1H, s), 9.00 (1H, s, NH).

(*Z*)-1-Acetyl-3-(2-benzyloxy-4,5-dimethoxy-3-methylphenylmethylene)-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (9b)——The same procedure as described above but using **4b** (57.2 mg, 0.2 mM) and **8**

(78.4 mg, 0.2 mM) afforded **9b** (104.1 mg, 84%) as colorless prisms, mp 142—143.5 °C. *Anal.* Calcd for $C_{34}H_{38}N_2O_9 \cdot 1/6C_6H_6$: C, 66.55; H, 6.22, N, 4.44. Found: C, 66.51; H, 6.28; N, 4.53. MS *m/z* (%): 618 (M^+, 14), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3305, 1705, 1645. UV λ_{max} nm (log ε): 258 (4.06), 294 (3.90), 336 (4.13). ^{1}H-NMR ($CDCl_3$) δ: 2.01 (3H, s, $ArCH_3$), 2.07 (3H, s, $ArCH_3$), 2.58 (3H, s, $COCH_3$), 2.86 (1H, dd, *J*=14, 3 Hz, ArCH), 3.19 (3H, s, OCH_3), 3.27 (1H, dd, *J*=14, 8 Hz, ArCH), 3.49 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 4.55 (2H, s, OCH_2), 5.27 (1H, dd, *J*=8, 3 Hz, CH), 6.13 (1H, s), 6.25 (1H, s), 6.46 (1H, s), 7.26 (5H, s, 5 × ArH), 8.88 (1H, s, NH).

(*Z*)-1-Acetyl-3-(5-benzyloxy-2,4-dimethoxy-3-methylphenylmethylene)-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (9c)——The same procedure as described above but using 5-benzyloxy-2,4-dimethoxy-3-methylbenzaldehyde (**4c**,[18] 143 mg, 0.5 mM) and **8** (196 mg, 0.5 mM) afforded **9c** (217.3 mg, 70%) as colorless prisms, mp 145—146.5 °C. *Anal.* Calcd for $C_{34}H_{38}N_2O_9$: C, 66.00; H, 6.19; N, 4.53. Found: C, 66.13; H, 6.26; N, 4.49. MS *m/z* (%): 618 (M^+, 14), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3230, 1705, 1690, 1640. UV λ_{max} nm (log ε): 256 (4.06), 294 (3.95),332 (4.16). ^{1}H-NMR ($CDCl_3$) δ: 2.05 (3H, s, $ArCH_3$), 2.16 (3H, s, $ArCH_3$), 2.58 (3H, s, $COCH_3$), 2.85 (1H, dd, *J*=14, 4 Hz, ArCH), 3.19 (3H, s, OCH_3), 3.40 (1H, dd, *J*=14, 6 Hz, ArCH), 3.46 (3H, s, OCH_3), 3.50 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 5.07 (1H, d, *J*=12 Hz, OCH), 5.34 (1H, dd, *J*=6, 4 Hz, CH), 5.39 (1H, d, *J*=12 Hz, OCH), 6.15 (1H, s), 6.23 (1H, s), 6.54 (1H, s), 7.24—7.60 (5H, m, 5 × ArH), 9.02 (1H, s, NH).

(*Z*)-1-Acetyl-3-(3-methoxy-4-methylphenylmethylene)-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (9d)——The same procedure as described above but using 3-methoxy-4-methylbenzaldehyde (**4d**,[19] 75 mg, 0.5 mM) and **8** (196 mg, 0.5 mM) afforded **9d** (140.7 mg, 58%) as colorless prisms, mp 129.5—131 °C. *Anal.* Calcd for $C_{26}H_{30}N_2O_7$: C, 64.71; H, 6.27; N, 5.81. Found: C, 64.96; H, 6.33; N, 5.81. MS *m/z* (%): 482 (M^+, 15), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3160, 1690, 1675, 1625. UV λ_{max} nm (log ε): 287 (3.91), 336 (4.20). ^{1}H-NMR ($CDCl_3$) δ: 1.99 (3H, s, $ArCH_3$), 2.17 (3H, s, $ArCH_3$), 2.57 (3H, s, $COCH_3$), 3.09 (1H, dd, *J*=14, 4 Hz, ArCH), 3.31 (1H, dd, *J*=14, 6 Hz, ArCH), 3.51 (3H, s, OCH_3), 3.55 (3H, s, OCH_3), 3.63 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 5.33 (1H, dd, *J*=6, 4 Hz, CH), 6.32 (1H, s), 6.44 (1H, dd, *J*=8, 2 Hz, ArH), 6.49 (1H, s), 6.61 (1H, d, *J*=2 Hz, ArH), 7.09 (1H, d, *J*=8 Hz, ArH), 7.67 (1H, s, NH).

(*Z*)-1-Acetyl-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,3,5-trimethoxy-4-methylphenylmethylene)-2,5-piperadinedione (9e)—— The same procedure as described above but using 2,3,5-trimethoxy-4-methylbenzaldehyde (**4e**,[7] 105 mg, 0.5 mM) and **8** (196 mg, 0.5 mM) afforded **9e** (210.3 mg, 78%) as colorless prisms, mp 137—138.5 °C. *Anal.* Calcd for $C_{28}H_{34}N_2O_9$: C, 61.98; H, 6.32; N, 5.16. Found: C, 62.06; H, 6.39; N, 5.12. MS *m/z* (%): 542 (M^+, 18), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3150, 1705, 1690, 1630. UV λ_{max} nm (log ε): 284 (3.89), 330 (4.17). ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s, $ArCH_3$), 2.12 (3H, s, $ArCH_3$), 2.59 (3H, s, $COCH_3$), 2.93 (1H, dd, *J*=14, 6 Hz, ArCH), 3.26 (3H, s, OCH_3), 3.51 (3H, s, OCH_3), 3.64 (3H, s, OCH_3), 3.66 (1H, dd, *J*=14, 6 Hz, ArCH), 3.66 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 5.29 (1H, dd, *J*=6, 4 Hz, CH), 6.20 (1H, s), 6.29 (1H, s), 6.31 (1H, s), 9.11 (1H, s, NH).

(*Z*)-1-Acetyl-3-(2-benzyloxy-3,5-dimethoxy-4-methylphenylmethylene)-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-2,5-piperazinedione (9f)——The same procedure as described above but using **4f** (143 mg, 0.5 mM) and **8** (196 mg, 0.5 mM) afforded **9f** (206.4 mg, 67%) as colorless prisms, mp 131—132.5 °C. *Anal.* Calcd for $C_{34}H_{38}N_2O_9$: C, 66.00; H, 6.19; N, 4.53. Found: C, 65.90; H, 6.33; N, 4.39. MS *m/z* (%): 618 (M^+, 15), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3160, 1700, 1680, 1630. UV λ_{max} nm (log ε): 329 (4.14). ^{1}H-NMR ($CDCl_3$) δ: 2.00 (3H, s, $ArCH_3$), 2.14 (3H, s, $ArCH_3$), 2.56 (3H, s, $COCH_3$), 2.89 (1H, dd, *J*=14, 4 Hz, ArCH), 3.26 (3H, s, OCH_3), 3.29 (1H, dd, *J*=14, 6 Hz, ArCH), 3.49 (3H, s, OCH_3), 3.64 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 4.59 (1H, d, *J*=12 Hz, OCH), 4.93 (1H, d, *J*=12 Hz, OCH), 5.26 (1H, dd, *J*=6, 4 Hz, CH), 6.17 (1H, s), 6.22 (1H, s), 6.26 (1H, s), 7.27 (5H, s, 5 × ArH), 8.87 (1H, s, NH).

(*Z*)-3-Arylidene-6-arylmethyl-2,5-piperazinediones 10: General Procedure——Hydrazine hydrate (10 μl) was added to a stirred solution of **9** (0.1 mM) in DMF (1 ml), and the resulting solution was stirred for 12 h at room temperature. The reaction mixture was poured into water (10 ml) and extracted with benzene. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give a solid, recrystallization of which from AcOEt–Et_2O gave **10** in 51—89% yield.

10a: Yield 89%, mp 152—153 °C. *Anal.* Calcd for $C_{26}H_{32}N_2O_8$: C, 62.39; H, 6.44; N, 5.60. Found: C, 62.43; H, 6.50; N, 5.56. MS *m/z* (%): 500 (M^+, 16), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3230, 1690, 1670, 1630. UV λ_{max} nm (log ε): 226 (4.34), 290 (4.17), 308 (4.22). ^{1}H-NMR ($CDCl_3$) δ: 2.18 (3H, s, $ArCH_3$), 2.22 (3H, s, $ArCH_3$), 2.94 (1H, dd, *J*=15, 8 Hz, ArCH), 3.35 (1H, dd, *J*=15, 4 Hz, ArCH), 3.59 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 4.42 (1H, m, CH), 6.35 (1H, s, NH), 6.54 (1H, s), 6.59 (1H, s), 6.69 (1H, s), 9.18 (1H, s, NH).

10b: Yield 51%, mp 132—134 °C. *Anal.* Calcd for $C_{32}H_{36}N_2O_8$: C, 66.65; H, 6.29; N, 4.86. Found: C, 66.81; H, 6.45; N, 4.77. MS *m/z* (%): 576 (M^+, 15), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3200, 1685, 1635. UV λ_{max} nm (log ε): 290 (4.13), 309 (4.19). ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, s, $ArCH_3$), 2.21 (3H, s, $ArCH_3$), 2.85 (1H, dd, *J*=14, 9 Hz, ArCH), 3.32 (1H, dd, *J*=14, 4 Hz, ArCH), 3.69 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 4.31 (1H, m, CH), 4.70 (2H, s, OCH_2), 6.09 (1H, s, NH), 6.52 (1H, s), 6.64 (1H, s), 6.77 (1H, s), 7.31 (5H, s, 5 × ArH), 8.96 (1H, s, NH).

10c: Yield 68%, mp 163—164 °C. *Anal.* Calcd for $C_{32}H_{36}N_2O_8 \cdot 1/4H_2O$: C, 66.14; H, 6.33; N, 4.82. Found: C, 65.99; H, 6.35; N, 4.76. MS *m/z* (%): 576 (M^+, 21), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3280, 3120, 1705, 1680, 1635. UV λ_{max} nm

(log ε): 290 (3.13), 308 (3.19). ^{1}H-NMR ($CDCl_3$) δ: 2.21 (3H, s, $ArCH_3$), 2.24 (3H, s, $ArCH_3$), 2.97 (1H, dd, J=14, 8 Hz, ArCH), 3.34 (1H, dd, J=14, 4 Hz, ArCH), 3.59 (3H, s, OCH_3), 3.69 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 4.44 (1H, m, CH), 5.07 (1H, d, J=12 Hz, OCH), 5.11 (1H, d, J=12 Hz, OCH), 6.15 (1H, s, NH), 6.55 (1H, s), 6.68 (1H, s), 6.69 (1H, s), 7.26—7.45 (5H, m, 5×ArH), 9.22 (1H, s, NH).

10d: Yield 77%, mp 200—201 °C. *Anal.* Calcd for $C_{24}H_{28}N_2O_6$: C, 65.44; H, 6.41; N, 6.36. Found: C, 65.35; H, 6.49; N, 6.23. MS *m/z* (%): 440 (M^+, 11), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3200, 1690, 1635. UV λ_{max} nm (log ε): 232 (4.25), 290 (4.13), 312 (4.19). ^{1}H-NMR ($CDCl_3$) δ: 2.20 (3H, s, $ArCH_3$), 2.23 (3H, s, $ArCH_3$), 3.02 (1H, dd, J=15, 8 Hz, ArCH), 3.38 (1H, dd, J=15, 4 Hz, ArCH), 3.74 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 4.48 (1H, m, CH), 6.50 (1H, s, NH), 6.59 (1H, s), 6.72 (1H, d, J=2 Hz, ArH), 6.82 (1H, dd, J=8, 2 Hz, ArH), 7.16 (1H, d, J=8 Hz, ArH), 7.95 (1H, s, NH).

10e: Yield 89%, mp 157—158 °C. *Anal.* Calcd for $C_{26}H_{32}N_2O_8$: C, 62.39; H, 6.44; N, 5.60. Found: C, 62.19; H, 6.55; N, 5.49. MS *m/z* (%): 500 (M^+, 13), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3220, 1690, 1665, 1635. UV λ_{max} nm (log ε): 236 (4.34), 290 (4.17), 307 (4.24). ^{1}H-NMR ($CDCl_3$) δ: 2.15 (3H, s, $ArCH_3$), 2.20 (3H, s, $ArCH_3$), 2.95 (1H, dd, J=15, 10 Hz, ArCH), 3.40 (1H, dd, J=15, 4 Hz, ArCH), 3.72 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 4.45 (1H, m, CH), 6.30 (1H, s, NH), 6.44 (1H, s), 6.57 (1H, s), 6.73 (1H, s), 9.28 (1H, s, NH).

10f: Yield 61%, mp 95—97 °C. *Anal.* Calcd for $C_{32}H_{36}N_2O_8$: C, 66.65; H, 6.29; N, 4.68. Found: C, 66.25; H, 6.29; N, 4.86. MS *m/z* (%): 576 (M^+, 17), 195 (100). IR ν_{max}^{KBr} cm^{-1}: 3190, 1685, 1670, 1635. UV λ_{max} nm (log ε): 299 (4.14), 307 (4.20). ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, s, $ArCH_3$), 2.20 (3H, s, $ArCH_3$), 2.85 (1H, dd, J=15, 10 Hz, ArCH), 3.33 (1H, dd, J=15, 4 Hz, ArCH), 3.72 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 4.32 (1H, m, CH), 4.90 (2H, s, OCH_2), 6.18 (1H, s, NH), 6.43 (1H, s), 6.54 (1H, s), 6.69 (1H, s), 7.32 (5H, s, 5×ArH), 8.98 (1H, s, NH).

Reaction of 10a with Acetic Anhydride——By the same procedure as used to prepare **8**, the reaction of **10a** (2.0 g, 4 mM) with acetic anhydride afforded **9a** (1.53 g, 71%). The spectral data were identical with those of the product obtained earlier.

Alkylation of 10a——Sodium hydride (50% oil dispersion, washed with dry hexane three times, 80 mg, 3.3 mM) was added to a stirred solution of **10a** (1.5 g, 3 mM) in dry DMF (25 ml) under ice-cooling, and stirring was continued for 10 min at 0 °C. Benzyl bromide (0.39 ml, 3.3 mM) in dry DMF (5 ml) was added. The reaction mixture was stirred for 2 h at room temperature, poured into ice-water, and extracted with benzene. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give the residue. Chromatography on silica gel column with benzene–AcOEt (4 : 1) as the eluent gave the dibenzyl derivative **13** (450 mg, 22%). Further elution with benzene–AcOEt (2 : 1—1 : 1) gave the *N*-benzyl derivative **11** (1.09 g, 61%). Finally, elution with AcOEt afforded the *O*-benzyl derivative **12** (141 mg, 8%).

(*Z*)-4-Benzyl-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-2,5-piperazinedione (**11**): mp 170—172 °C (colorless prisms from acetone). *Anal.* Calcd for $C_{33}H_{38}N_2O_8$: C, 67.10; H, 6.49; N, 4.74. Found: C, 66.95; H, 6.52; N, 4.67. MS *m/z* (%): 590 (M^+, 13), 560 (37), 559 (100), 195 (79), 91 (31). IR ν_{max}^{KBr} cm^{-1}: 3170, 1675, 1620. UV λ_{max} nm (log ε): 290 (4.08), 320 (4.10), 332 (4.09). ^{1}H-NMR ($CDCl_3$) δ: 2.21 (3H, s, $ArCH_3$), 2.23 (3H, s, $ArCH_3$), 3.04 (1H, dd, J=14, 9 Hz, ArCH), 3.41 (1H, dd, J=14, 4 Hz, ArCH), 3.53 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.80 (6H, s, 2×OCH_3), 3.86 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 4.38 (1H, m, CH), 4.59 (1H, d, J=14 Hz, NCH), 4.94 (1H, d, J=14 Hz, NCH), 6.54 (1H, s, NH), 6.62 (2H, s), 6.80—6.96 (2H, m, 2×ArH), 7.10 (1H, s), 7.04—7.20 (3H, m, 3×ArH). ^{13}C-NMR ($CDCl_3$) δ: 9.4 (q), 9.7 (q), 32.6 (t), 47.5 (t), 55.9 (d), 55.9 (q), 56.1 (q), 60.1 (q), 60.4 (q), 60.7 (q), 61.2 (q), 110.6 (d), 111.7 (d), 117.9 (d), 121.7 (s), 123.5 (s), 125.9 (s), 125.9 (s), 127.2 (d), 127.2 (d), 128.4 (d), 129.7 (s), 136.4 (s), 147.4 (s), 148.9 (s), 149.2 (s), 149.5 (s), 150.8 (s), 152.0 (s), 164.7 (s, CO), 167.4 (s, CO).

(*Z*)-5-Benzyloxy-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-1,2,3,6-tetrahydropyrazin-2-one (**12**): mp 207—209 °C (pale yellow needles from CH_2Cl_2–AcOEt). *Anal.* Calcd for $C_{33}H_{38}N_2O_8 \cdot 1/3H_2O$: C, 66.43; H, 6.53; N, 4.69. Found: C, 66.38; H, 6.44; N, 4.63. MS *m/z* (%): 590 (M^+, 74), 499 (31), 195 (100), 182 (57), 91 (53). IR ν_{max}^{KBr} cm^{-1}: 3150, 1660, 1650. UV λ_{max} nm (log ε): 240 (4.07), 288 (3.92), 301 (4.01), 319 (4.11), 339 (4.18). ^{1}H-NMR ($CDCl_3$) δ: 2.10 (3H, s, $ArCH_3$), 2.21 (3H, s, $ArCH_3$), 2.96 (1H, dd, J=14, 7 Hz, ArCH), 3.20 (1H, dd, J=14, 5 Hz, ArCH), 3.64 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 4.57 (1H, m, CH), 5.35 (1H, d, J=13 Hz, NCH), 5.46 (1H, d, J=13 Hz, NCH), 6.52 (1H, s, NH), 6.53 (1H, s), 7.40 (5H, s, 5×ArH), 7.42 (1H, s), 8.14 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 9.5 (q), 9.7 (q), 36.4 (t), 54.0 (d), 55.8 (q), 56.1 (q), 60.2 (q), 60.3 (q), 60.6 (q), 62.0 (q), 68.6 (t, OCH_2), 111.9 (d), 112.1 (d), 119.9 (d), 122.7 (s), 123.5 (s), 125.1 (s), 126.1 (s), 127.8 (d), 128.4 (d), 128.7 (d), 129.7 (s), 135.8 (s), 147.7 (s), 148.6 (s), 148.8 (s), 149.3 (s), 151.3 (s), 153.2 (s), 160.4 (s, N=C–O), 162.4 (CO).

(*Z*)-1,4-Dibenzyl-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-2,5-piperazinedione (**13**): Amorphous powder. MS *m/z* (%): 680 (M^+, 10), 649 (96), 195 (100), 91 (72). High-resolution MS Calcd for $C_{40}H_{44}N_2O_8$: 680.3097. Found: 680.3080. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1675. UV λ_{max} nm: 289, 330. ^{1}H-NMR ($CDCl_3$) δ: 2.11 (3H, s, $ArCH_3$), 2.23 (3H, s, $ArCH_3$), 3.16 (2H, d, J=7 Hz, $ArCH_2$), 3.35 (1H, d, J=14 Hz, NCH), 3.60 (3H, s, OCH_3), 3.66 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.91

(3H, s, OCH_3), 4.16 (1H, d, J=14 Hz, NCH), 4.28 (1H, t, J=7 Hz, CH), 5.26 (1H, d, J=14 Hz, NCH), 5.30 (1H, d, J=14 Hz, NCH), 6.48 (1H, s), 6.72 (1H, s), 6.80—7.06 (4H, m, 4×ArH), 7.19—7.28 (6H, m, 6×ArH), 7.21 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 9.4 (q), 9.7 (q), 33.0 (t), 47.8 (t), 48.3 (t), 56.1 (d), 56.2 (q), 60.1 (q), 60.4 (q), 60.9 (q), 61.3 (q), 61.7 (q), 110.6 (d), 111.7 (d), 117.3 (d), 121.7 (s), 123.2 (s), 125.8 (s), 125.9 (s), 127.4 (d), 127.5 (d), 127.8 (d), 127.8 (d), 128.4 (d), 128.4 (d), 128.7 (s), 128.7 (s), 129.6 (s), 136.0 (s), 136.6 (s), 149.0 (s), 149.3 (s), 151.1 (s), 152.2 (s), 163.3 (s, CO), 167.4 (s, CO).

Acetylation of the Monobenzyl Derivatives 11 and 12——The monobenzyl derivative **11** (or **12**) (29.5 mg, 0.05 mM) was converted to the 1-acetyl derivative **15** (or **14**) in quantitative yield by the same procedure as that used for conversion of **7** to **8**.

(*Z*)-1-Acetyl-4-benzyl-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-2,5-piperazinedione (**15**): Amorphous powder. MS m/*z* (%): 632 (M^+, 23), 559 (13), 195 (100), 91 (15). High-resolution MS Calcd for $C_{35}H_{40}N_2O_9$: 632.2733. Found: 632.2758. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1700, 1685, 1620. UV λ_{max} nm (log ε): 250 (3.97), 288 (3.78), 344 (3.97). ^{1}H-NMR ($CDCl_3$) δ: 1.96 (3H, s, $ArCH_3$), 2.20 (3H, s, $ArCH_3$), 2.54 (3H, s, $COCH_3$), 3.21 (2H, d, J=7 Hz, $ArCH_2$), 3.50 (3H, s, OCH_3), 3.60 (3H, s, OCH_3), 3.62 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.90 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 4.22 (1H, d, J=14 Hz, NCH), 5.35 (1H, d, J=14 Hz, NCH), 5.50 (1H, t, J=7 Hz, CH), 6.50 (1H, s), 6.86 (1H, s), 6.80—6.92 (2H, m, 2×ArH), 7.14—7.21 (3H, m, 3×ArH).

(*Z*)-1-Acetyl-5-benzyloxy-6-(2,4,5-trimethoxy-3-methylphenylmethyl)-3-(2,4,5-trimethoxy-3-methylphenylmethylene)-1,2,3,6-tetrahydropyrazin-2-one (**14**): mp 121—123 °C (pale yellow needles from Et_2O). *Anal.* Calcd for $C_{35}H_{40}N_2O_9$: C, 66.44; H, 6.37; N, 4.43. Found: C, 66.26; H, 6.29; N, 4.41. MS *m*/*z* (%): 632 (M^+, 12), 195 (100), 91 (13). IR ν_{max}^{KBr} cm^{-1}: 1690, 1650. UV λ_{max} nm (log ε): 244 (4.17), 288 (3.83), 312 (4.01), 356 (4.29). ^{1}H-NMR ($CDCl_3$) δ: 1.83 (3H, s, $ArCH_3$), 2.18 (3H, s, $ArCH_3$), 2.66 (3H, s, $COCH_3$), 3.01 (1H, dd, J=14, 6 Hz, ArCH), 3.22 (1H, dd, J=14, 4 Hz, ArCH), 3.49 (3H, s, OCH_3), 3.56 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 5.22 (1H, d, J=13 Hz, NCH), 5.41 (1H, dd, J=6, 4 Hz, CH), 5.44 (1H, d, J=13 Hz, NCH), 6.30 (1H, s), 7.17 (1H, s), 7.40 (5H, s, 5×ArH).

Benzylation of the *N*-Benzyl Derivative 11——Sodium hydride (2.8 mg, 0.12 mM) was added to a stirred solution of **11** (59 mg, 0.1 mM) in dry DMF (1 ml) under ice-cooling, and stirring was continued for 10 min at 0 °C. Benzyl bromide (14 μl, 0.12 mM) in dry DMF (1 ml) was added. The reaction mixture was stirred for 30 min at room temperature, poured into ice-water and extracted with $CHCl_3$. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give the residue, which was purified by column chromatography on silica gel with benzene–AcOEt (10:1—8:1) as the eluent to give **13** (48 mg, 70%), whose spectra were identical with those of an authentic sample obtained earlier.

Benzylation of the *O*-Benzyl Derivative 12——The same procedure as described above but using **12** (32 mg, 0.054 mM) afforded **18** (25 mg, 67%) as yellow oil. MS *m*/*z* (%): 680 (M^+, 51), 590 (18), 589 (47), 195 (16), 91 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1490, 1455, 1415, 1355, 1320. UV λ_{max} nm (log ε): 238 (4.20), 284 (3.76), 322 (4.11). ^{1}H-NMR ($CDCl_3$) δ: 2.20 (6H, s, 2×$ArCH_3$), 3.66 (6H, s, 2×OCH_3), 3.69 (6H, s, 2×OCH_3), 3.78 (6H, s, 2×OCH_3), 4.07 (4H, s, 2×$ArCH_2$), 5.28 (4H, s, 2×OCH_2), 6.57 (2H, s, 2×ArH), 7.25 (10H, s, 10×ArH).

2-Benzyloxy-3,6-bis(2,4,5-trimethoxy-3-methylphenylmethyl)-5-hydroxypyrazine (16)——A catalytic amount of KOH pellets was added to a stirred solution of **12** (21.8 mg, 0.037 mM) in methanol (3 ml), and the resulting solution was stirred for 3 h at room temperature. The reaction mixture was poured into water, acidified with 2 N HCl and extracted with $CHCl_3$. The extract was washed with brine, dried over Na_2SO_4, and evaporated to give the residue, which was purified by column chromatography on silica gel to give **16** (21 mg, 96%) as a pale yellow oil. MS *m*/*z* (%): 590 (M^+, 72), 499 (32), 467 (24), 377 (22), 195 (100), 182 (59), 91 (55). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3200—3000. UV λ_{max} nm (log ε): 232 (4.22), 282 (3.68), 366 (3.45). ^{1}H-NMR ($CDCl_3$) δ: 2.16 (3H, s, $ArCH_3$), 2.19 (3H, s, $ArCH_3$), 3.60 (3H, s, OCH_3), 3.62 (3H, s, OCH_3), 3.64 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 3.87 (2H, s, $ArCH_2$), 4.02 (2H, s, $ArCH_2$), 5.16 (2H, s, OCH_2), 6.46 (1H, s, ArH), 6.67 (1H, s, ArH), 7.08—7.44 (6H, m, 5×ArH and OH).

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A New Iridoid Glucoside, Nepetariaside, from *Nepeta cataria*[1)]

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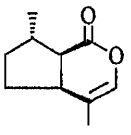
A new iridoid glucoside, named nepetariaside, was isolated from *Nepeta cataria* L. and its absolute structure was elucidated by chemical, spectral and X-ray crystallographic analysis.

Keywords——*Nepeta cataria*; Lamiaceae; iridoid glucoside; nepetariaside; X-ray analysis; 1,5,9-epideoxyloganic acid

Catnip, *Nepeta cataria* L. (Lamiaceae), is a plant indigenous to Europe, and is known to contain steam-volatile iridoid lactones, nepetalactone (**1**), dihydronepetalactone (**2**) and isodihydronepetalactone (**3**).[2,3)] Recently, we isolated an iridoid glucoside, 1,5,9-epideoxyloganic acid (**4**), from this plant and elucidated its structure.[4)] This paper deals with the isolation and structure elucidation of a further iridoid glucoside, named nepetariaside (**5**) which occurs in the same plant.

The methanolic extract of *Nepeta cataria* L. was successively fractionated by charcoal and silica gel column chromatography to give the new iridoid glucoside nepetariaside (**5**), along with 1,5,9-epideoxyloganic acid (**4**). Nepetariaside (**5**) was obtained as colorless needles, $C_{16}H_{28}O_8$, mp 139—141 °C (dec), $[\alpha]_D$ −14.0° (MeOH). It showed characteristic infrared (IR) bands at 3450, 3300, 3150—2250, and 1695 cm^{-1} due to hydroxy groups and a



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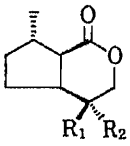

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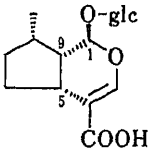

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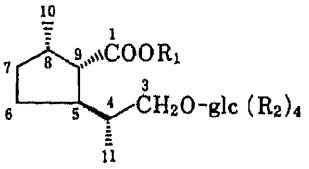
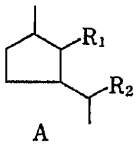
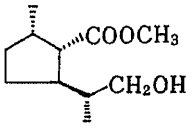


5 : R_1=R_2=H
6 : R_1=CH_3, R_2=H
7 : R_1=CH_3, R_2=Ac



A

R_1=COOH, R_2=CH_2OH
or
R_1=CH_2OH, R_2=COOH



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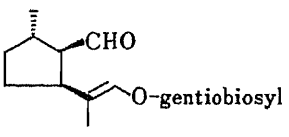

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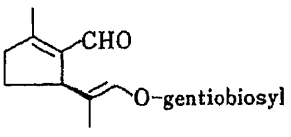
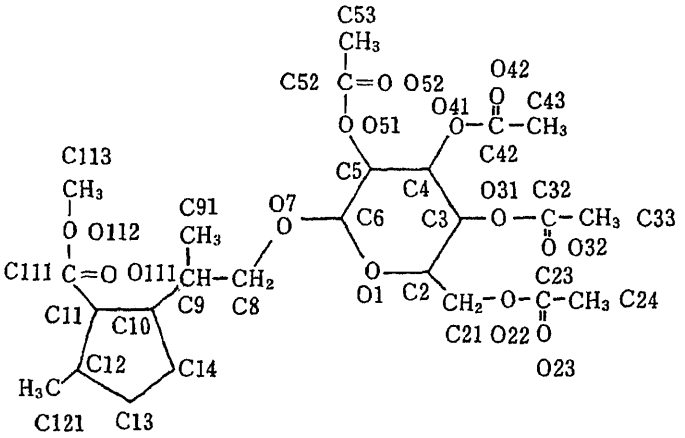


10

Fig. 1



atomic numbering system used in the X-ray analysis

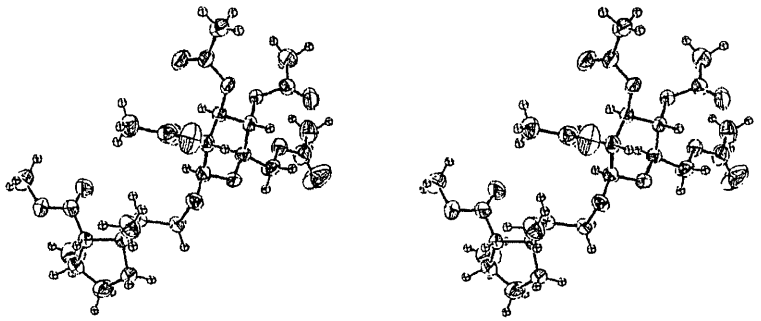


Fig. 2. Stereoscopic View of the Molecule of Nepetariaside Methyl Ester Acetate (7)

TABLE I. Nonhydrogen Atomic Coordinates of 7, along with Their Estimated Standard Deviations in Parentheses

Atom	x	y	z	Atom	x	y	z
O1	0.9196 (2)	0.2805 (4)	0.6293 (2)	C52	0.7082 (4)	0.8061 (7)	0.4703 (3)
C2	0.8637 (4)	0.2146 (6)	0.6702 (3)	O52	0.8049 (3)	0.8899 (5)	0.5263 (2)
C21	0.9732 (4)	0.0980 (7)	0.7492 (3)	C53	0.5935 (5)	0.8673 (8)	0.3760 (3)
O22	0.9211 (3)	0.0060 (4)	0.7876 (2)	C6	0.8264 (3)	0.3759 (6)	0.5461 (2)
C23	1.0020 (5)	−0.1051 (8)	0.8581 (3)	O7	0.8879 (3)	0.4256 (5)	0.5096 (2)
O23	1.1134 (4)	−0.1246 (9)	0.8884 (3)	C8	0.8731 (4)	0.2963 (7)	0.4458 (3)
C24	0.9389 (5)	−0.1844 (9)	0.8958 (3)	C9	0.7595 (4)	0.3526 (7)	0.3465 (2)
C3	0.8287 (3)	0.3799 (6)	0.7006 (2)	C91	0.7886 (6)	0.5352 (8)	0.3247 (4)
O31	0.7600 (2)	0.3261 (4)	0.7350 (2)	C10	0.7284 (4)	0.1991 (7)	0.2810 (2)
C32	0.8284 (4)	0.3288 (7)	0.8276 (3)	C11	0.6208 (4)	0.2381 (7)	0.1772 (3)
O32	0.9423 (3)	0.3647 (6)	0.8818 (2)	C111	0.4844 (4)	0.2536 (8)	0.1482 (3)
C33	0.7375 (5)	0.2866 (9)	0.8492 (3)	O111	0.4497 (3)	0.2027 (7)	0.1905 (2)
C4	0.7324 (3)	0.4974 (6)	0.6177 (2)	O112	0.4052 (3)	0.3296 (7)	0.0653 (2)
O41	0.7146 (2)	0.6606 (4)	0.6517 (2)	C113	0.2646 (5)	0.3332 (12)	0.0241 (7)
C42	0.5906 (4)	0.7243 (6)	0.6055 (3)	C12	0.6327 (5)	0.0804 (8)	0.1273 (3)
O42	0.4969 (3)	0.6574 (5)	0.5360 (2)	C121	0.5456 (7)	−0.0827 (11)	0.1073 (5)
C43	0.5906 (4)	0.8857 (7)	0.6527 (3)	C13	0.7790 (5)	0.0267 (9)	0.1953 (4)
C5	0.7885 (3)	0.5460 (6)	0.5695 (2)	C14	0.8424 (4)	0.1372 (8)	0.2829 (3)
O51	0.6874 (2)	0.6350 (4)	0.4842 (2)				

TABLE II. Bond Lengths (Å) of 7 with Their Standard Deviations

Atom	Atom	Distance	Atom	Atom	Distance
O1	C2	1.433 (6)	O1	C6	1.418 (6)
C2	C21	1.521 (7)	C2	C3	1.534 (7)
C21	O22	1.432 (7)	O22	C23	1.332 (7)
C23	O23	1.195 (9)	C23	C24	1.499 (10)
C3	O31	1.448 (5)	C3	C4	1.511 (6)
O31	C32	1.355 (6)	C32	O32	1.191 (7)
C32	C33	1.509 (9)	C4	O41	1.448 (5)
C4	C5	1.517 (6)	O41	C42	1.357 (6)
C42	O42	1.195 (6)	C42	C43	1.483 (7)
C5	O51	1.437 (5)	C5	C6	1.514 (6)
O51	C52	1.354 (6)	C52	O52	1.189 (7)
C52	C53	1.493 (8)	C6	O7	1.391 (6)
O7	C8	1.441 (6)	C8	C9	1.532 (7)
C9	C91	1.527 (9)	C9	C10	1.523 (7)
C10	C11	1.543 (7)	C10	C14	1.539 (8)
C11	C111	1.509 (8)	C11	C12	1.559 (8)
C111	O111	1.190 (8)	C111	O112	1.328 (8)
O112	C113	1.481 (13)	C12	C121	1.537 (10)
C12	C13	1.542 (9)	C13	C14	1.523 (8)

TABLE III. Bond Angle (°) of 7 with Their Standard Deviations

Atom	Atom	Atom	Angle	Atom	Atom	Atom	Angle
C2	O1	C6	112.0 (3)	O1	C2	C21	102.9 (4)
O1	C2	C3	107.2 (4)	C21	C2	C3	114.4 (4)
C2	C21	O22	107.4 (4)	C21	O22	C23	117.0 (4)
O22	C23	O23	121.1 (6)	O22	C23	C24	112.0 (5)
O23	C23	C24	126.7 (7)	C2	C3	O31	110.8 (4)
C2	C3	C4	109.4 (4)	O31	C3	C4	105.1 (3)
C3	O31	C32	117.5 (4)	O31	C32	O32	123.8 (5)
O31	C32	C33	109.7 (5)	O32	C32	C33	126.5 (5)
C3	C4	O41	107.1 (3)	C3	C4	C5	109.8 (4)
O41	C4	C5	109.3 (3)	C4	O41	C42	118.0 (3)
O41	C42	O42	123.1 (4)	O41	C42	C43	110.5 (4)
O42	C42	C43	126.4 (5)	C4	C5	O51	108.2 (3)
C4	C5	C6	109.9 (4)	O51	C5	C6	107.6 (4)
C5	O51	C52	119.0 (4)	O51	C52	O52	123.4 (5)
O51	C52	C53	109.6 (4)	O52	C52	C53	127.0 (5)
O1	C6	C5	108.2 (4)	O1	C6	O7	107.6 (4)
C5	C6	O7	108.4 (4)	C6	O7	C8	114.1 (4)
O7	C8	C9	109.9 (4)	C8	C9	C91	110.3 (5)
C8	C9	C10	109.4 (4)	C91	C9	C10	115.6 (5)
C9	C10	C11	116.0 (4)	C9	C10	C14	115.8 (4)
C11	C10	C14	101.1 (4)	C10	C11	C111	113.9 (4)
C10	C11	C12	104.7 (4)	C111	C11	C12	112.4 (5)
C11	C111	O111	126.5 (6)	C11	C111	O112	109.4 (5)
O111	C111	O112	124.1 (6)	C111	O112	C113	114.7 (6)
C11	C12	C121	113.3 (5)	C11	C12	C13	103.9 (5)
C121	C12	C13	110.2 (5)	C12	C13	C14	107.5 (5)
C10	C14	C13	105.6 (5)				

carboxy group, whereas no absorption maximum was observed in the ultraviolet (UV) region above 210 nm. Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum (100 MHz, CD_3OD) showed signals due to two methyl groups (C(10) and C(11)) at δ 0.92 and δ 0.97 ppm (each d, J=7.0 Hz), and a signal due to an oxygen-bearing methylene group (C(3) or C(1)) at 4.22 ppm (d, J=8.0 Hz), as well as resonances arising from a sugar moiety. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) (25 MHz, CD_3OD) spectrum showed signals due to two methyl groups at δ 14.68 and δ 16.73 ppm, and two methylenes at δ 29.37 and δ 35.16 ppm, which suggest the presence of unsubstituted methylenes at C(6) and C(7), in addition to the signals of an oxygen-bearing methylene group at δ 75.18 and a carboxy carbon at δ 179.31 ppm. It further showed the carbon signals of a β-glucopyranosyloxy group.[5]

Nepetariaside (**5**) gave the methyl ester (**6**) on methylation and the latter afforded the methyl ester tetraacetate (**7**) on acetylation. On enzymatic hydrolysis with β-glucosidase, the methyl ester (**6**) gave the aglucone methyl ester (**8**) and D-glucose.

On the basis of the above results it was inferred that the structure of the aglucone moiety of the glucoside (**5**) is shown by formula A,[8] with the stereochemistry still to be elucidated. Finally, the absolute structure of nepetariaside (**5**) was determined through a single crystal X-ray analysis by taking account of the absolute structure of the D-glucose moiety. The molecular structure of nepetariaside methyl ester tetraacetate (**7**) thus determined is shown in Fig. 2. The final atomic parameters are given in Table I. The bond lengths and angles lie in the normal ranges, as shown in Tables II and III.

Nepetariaside (**5**), possessing an aglucone with an opened dihydropyran ring, belongs to the same type of glycosides as iridodialogentiobioside (**9**)[6] and dehydroiridodialogentiobioside (**10**),[7] which were isolated from *Actinidia polygama* MIQ. It is noteworthy that the configurations at C-5 and C-9 of nepetariaside (**5**), 1,5,9-epideoxyloganic acid (**4**) and lactones such as nepetalactone (**1**) are different. This is the first example of the cooccurrence of such iridoids in the same plant.

Experimental

All melting points were determined on a Thomas Hoover apparatus and are uncorrected. The IR spectra were recorded with a JASCO IR-G spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on JNM-MH-100 and JNM-FX-100 spectrometers in CD_3OD or $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. Optical rotations were determined on a JASCO DIP-180 digital polarimeter. Gas-liquid chromatography (GLC) analyses were performed on a NEVA model 1700 gas chromatograph (column, 5′ × 1/4′, 20% Apiezon L on a Chromosorb W 100—120 mesh) under the following conditions: flow rate 17 ml/min (He); column temperature 180 °C; injection block temperature, 200 °C. Silica gel (Fuji Davison Chemical Ltd.) or carbon for chromatography (Wako) was used for column chromatography. For thin layer chromatography (TLC) Silica gel 60 F_{254} (Merck) was used, and spots were detected by spraying the plates with anisaldehyde–H_2SO_4 reagent followed by heating.

Isolation of Nepetariaside (5) and 1,5,9-Epideoxyloganic Acid (4)——Fresh leaves and stems of *Nepeta cataria* L. (15 kg) cultivated in the campus of Aichi Medical University were collected in July and extracted with MeOH (15 l × 3) under reflux for 1 h. The MeOH extract was evaporated *in vacuo* to give a brown mass, which was dissolved in H_2O and washed with Et_2O. The aqueous layer was concentrated *in vacuo* to a small volume and shaken with BuOH. The BuOH layer was concentrated *in vacuo* to give a residue (90.5 g), which was subjected to charcoal (500 g) column chromatography, successively eluting with H_2O (10 l) and MeOH (10 l). The residue (20.5 g) obtained through concentration of the MeOH eluate was applied to a column of silica gel (200 g) and eluted with mixtures of $CHCl_3$–EtOH containing increasing amounts of EtOH: fr. 1, 3.8 g, eluted with $CHCl_3$–EtOH (23 : 2) and fr. 2, 9.1 g, eluted with $CHCl_3$–EtOH (22 : 3 to 9 : 1). Fr. 1 was again chromatographed on silica gel (60 g) with the same $CHCl_3$–EtOH system, and the eluates with $CHCl_3$–EtOH (23 : 2) showing a spot of *Rf* 0.65 ($CHCl_3$–EtOH, 3 : 1) on TLC were combined and concentrated *in vacuo*. The residue (2.2 g) was rechromatographed on a silica gel (60 g) column with the same solvent system; the eluate with $CHCl_3$–EtOH (23 : 2) afforded a crystalline substance (960 mg) which, after being washed with Me_2CO and recrystallized from AcOEt, gave colorless needles (300 mg) of nepetariaside (**5**), mp 139—141 °C (dec.), $[\alpha]_D^{28}$ −14.0° (c=1.7, MeOH). IR ν_{max}^{Nujol}: 3450, 3300, 3150—2250, 1695 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.92 (3H, d, J=7.0 Hz, H_3(10)), 0.97 (3H, d, J=7.0 Hz, H_3(11)), 4.22 (2H, d, J=8.0 Hz, H(3)). ^{13}C-NMR (CD_3OD) δ: 179.31 (s, C(1)), 75.18 (t, C(3)), 38.55 (d, C(4)), 45.69 (d, C(5)), 29.37 (t, C(6)), 35.16 (t, C(7)), 38.55 (d, C(8)), 53.24

(d, C(9)), 16.73 (q, C(10)), 14.68 (q, C(11)), 104.37 (d, C(1')), 75.06 (d, C(2')), 77.75 (d, C(3') or C(5')), 71.55 (d, C(4')), 77.92 (d, C(5') or C(3')), 62.72 (t, C(6')). *Anal.* Calcd for $C_{16}H_{28}O_8$: C, 55.16; H, 8.10. Found: C, 54.89; H, 8.43.

Fr. 2 was worked up in the same way as reported previously[4] to give colorless needles (6.5 g) of 1,5,9-epideoxyloganic acid (**4**), mp 106 °C, $[\alpha]_D^{28}$ +85.1° (c=1.1, MeOH).

Nepetariaside Methyl Ester (6)——Nepetariaside (**5**) (110 mg) was methylated in a MeOH solution with ethereal CH_2N_2 to afford **6** as a viscous syrup (96 mg). $[\alpha]_D^{23}$ −16.5° (c=1.5, MeOH). IR ν_{max}^{Nujol}: 3400, 1730 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.92 (3H, d, J=7.0 Hz, H_3(10)), 0.97 (3H, d, J=7.0 Hz, H_3(11)), 3.68 (3H, s, $COOCH_3$), 4.20 (2H, d, J=8.0 Hz, H(3)).

Nepetariaside Methyl Ester Tetraacetate (7)——Nepetariaside methyl ester (**6**) (40 mg) was acetylated with Ac_2O–pyridine (each 1 ml) in the usual manner. The product was recrystallized from MeOH to afford **7** as colorless needles (47 mg). mp 98—99 °C, $[\alpha]_D^{23}$ −13.5° (c=1, $CHCl_3$). IR ν_{max}^{Nujol}: 1755, 1705 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, d, J=7.0 Hz, H_3(10)), 0.91 (3H, d, J=7.0 Hz, H_3(11)), 1.96—2.04 (12H, m, 4×$OCOCH_3$), 3.59 (3H, s, $COOCH_3$), 4.38 (1H, d, J=8.0 Hz, H(1')), 4.80—5.12 (4H, m, axial protons of glucose moiety). *Anal.* Calcd for $C_{25}H_{38}O_{12}$: C, 56.59; H, 7.22. Found: C, 56.48; H, 7.49.

Enzymatic Hydrolysis of Nepetariaside Methyl Ester (6)——The methyl ester (**6**) (326 mg) was hydrolyzed with β-glucosidase (20 mg, from almond, Sigma Chemical Co.) in a 0.1 M acetate buffer solution (pH 5.0, 4 ml) in the usual manner. The reaction mixture was extracted with ether and the ethereal layer was dried over Na_2SO_4 and concentrated. The residue was subjected to silica gel (3 g) column chromatography with C_6H_6 to give **8** as a colorless oily substance (134 mg), which showed a single spot of Rf 0.8 (C_6H_6–AcOEt, 1:1) on TLC. $[\alpha]_D^{23}$ +7.5° (c=1.3, $CHCl_3$). IR ν_{max}^{neat}: 3350, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.90 (3H, d, J=7.0 Hz, H_3(10)), 0.97 (3H, d, J=7.0 Hz, H_3(11)), 3.68 (3H, s, $COOCH_3$). *Anal.* Calcd for $C_{11}H_{20}O_3 \cdot 3/4H_2O$: C, 61.80; H, 10.13. Found: C, 62.06; H, 10.01.

The aqueous layer was concentrated *in vacuo* to give a residue which showed a spot of glucose on TLC (Rf 0.44, $CHCl_3$–MeOH–H_2O, 6:4:1).

X-Ray Analysis of Nepetariaside Methyl Ester Tetraacetate (7)——A crystal of **7** of approx. 0.3×0.4×0.2 mm was used for the X-ray study. Preliminary oscillation and Weissenberg photographs unequivocally showed the crystal to be monoclinic with space group $P2_1$ from the systematic absences. Crystal data determined on a Rikagaku four-circle diffractometer with graphite-monochromated CuK_α radiation and refined by the least-squares method are as follows: a=13.078(6), b=7.401(3), c=18.627(9) Å, β=128.93(2)°, V=1403(1) Å^3, Z=2, D_x=1.256 g cm^{-3}. To determine the structure, 2595 independent intensities (2θ less than 130°) were measured on the same diffractometer employing the $\omega-2\theta$ scan technique with the scan speed of 4° min^{-1}, and were corrected for Lorentz and polarization factors, but not for absorption effect because of the small size of the crystal used. The intensities of four standard reflections measured at every 100 reflections indicated no structural deterioration during the data collection. The structure was solved by the direct method (with MULTAN[9]) and refined by the block-diagonal least-squares method with anisotropic temperature fractors for nonhydrogen atoms and with isotropic ones for hydrogen atoms. The final R (R_w) value was 0.047 (0.044). The final atomic coordinates and bond lengths and angles between nonhydrogen atoms are summarized in Tables I—III.

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Metabolism and Cytotoxicity of Hydrazine in Isolated Rat Hepatocytes[1)]

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The relationship between the metabolism and hepatotoxicity of hydrazine (Hz) was studied in an isolated rat hepatocyte system. After incubation at 37 °C for 60 min, the concentration of Hz decreased to 34% of the initial value (from 98 to 33 nmol/ml). A further pronounced loss was observed when Hz was incubated with hepatocytes isolated from rats which had been pretreated with phenobarbital (PB) or rifampicin. Since metyrapone and piperonylbutoxide inhibited the metabolism in the systems obtained from both PB-pretreated rats and untreated rats, an important role of hepatic cytochrome P-450 in Hz oxidation was suggested. The difference spectrum of Hz with cytochrome P-450 in isolated rat hepatocytes demonstrated the formation of complexes between metabolic intermediates of Hz and cytochrome P-450. The trypan blue exclusion test, the measurement of K^+ level and the determination of glutathione content retained in the cells were also performed. The results suggested that Hz is cytotoxic to isolated rat hepatocytes.

Keywords——hydrazine; metabolism; cytotoxicity; hepatocytes; rat

Hydrazine (Hz) is a toxic and hazardous metabolite of isoniazid (INH) causing fatty liver and liver necrosis, and it is also a mutagen and a carcinogen.[2,3)] It has been detected by gas chromatography–mass spectrometry (GC-MS) in the urine of INH-dosed patients with tuberculosis.[4)] It is also well-known that liver damage is frequently induced in patients on INH treatment. Thus, the toxicity of Hz and its derivatives is of great interest from the point of view of the side effects in INH therapy. Although the exact sequence of events leading ultimately to liver injury has not been fully elucidated, Timbrell *et al.* postulated that INH-induced liver necrosis may be caused by a chemically reactive metabolite of INH, monoacetylhydrazine (AcHz).[5)] On the other hand, we found that Hz induced more marked hepatic injury than AcHz, and analogous but more extensive necrosis took place after Hz administration to rabbits pretreated with an inducer of cytochrome P-450 [phenobarbital (PB) or rifampicin (RMP)].[6)] Liver function tests and histological studies indicated similar liver injury in rats to that in rabbits.[7)] We also found that the liver and plasma concentrations of Hz in both animals after the intraperitoneal injection of INH were almost the same as those of AcHz.[8)] In the pretreated rats, the liver and plasma Hz concentrations were considerably lower than those of the control group, but this was not the case with AcHz.

These facts prompted us to compare the cytotoxicities of Hz and AcHz using an isolated rat hepatocyte system. The previous experiments showed that the incubation of Hz and AcHz in isolated rat hepatocytes induced marked concentration-dependent cell death, as indicated by a trypan blue exclusion test.[9)] This test did not show a distinct difference between Hz and AcHz in terms of the extent of cell injury. However, our preliminary investigations have also

shown that a significant time- and concentration-dependent depletion of hepatocellular reduced glutathione (GSH) levels was induced by Hz treatment, while AcHz caused very little GSH depletion.[9]

The data suggest that a key step of Hz metabolism produces the toxic intermediate(s). As the ultimate metabolite of Hz is known to be nitrogen (N_2),[10] the induction of hepatotoxicity by Hz may be due to a metabolic intermediate formed during the microsomal oxidation process. Recently, we reported that Hz metabolism in rat liver microsomal fractions resulted in the formation of Hz radical and diimide.[11,12] Therefore, in order to clarify the relationship of Hz metabolism to its hepatotoxicity, the present examination was designed to explore the toxic effects of Hz on isolated rat cells.

Materials and Methods

Chemicals——All chemicals were commercial products of reagent grade.

Animal Treatment——Male Wistar rats weighing 250—300 g were used. As for PB-pretreated animals, sodium PB was given intraperitoneally (i.p.) at a daily dose of 50 mg/kg for 3 d. In the case of RMP-pretreated animals, 30 mg/kg of RMP was given i.p. in 5 ml of HCl solution (pH 3.0). The control group received the vehicle only.

Preparation and Incubation of Isolated Rat Hepatocytes——The hepatocytes were isolated by the collagenase perfusion method according to the previously reported protocols.[9] The yield of each preparation was $2—4 \times 10^8$ cells/liver. Viability of the cells was determined by means of the trypan blue exclusion test.[13] Hepatocytes with a viability of more than 95% were used for the experiments. The isolated cells were suspended in Krebs-Henseleit buffer (pH 7.4) containing 1% bovine serum albumin, 10 mM glucose and 13 mM Hepes. Then 4×10^6 cells/ml were incubated at 37 °C in rotating round-bottomed flasks under an atmosphere of 95% O_2–5% CO_2. Prior to the addition of the test compounds, the cells were allowed to equilibrate for 3 min at 37 °C.

Hz Metabolism in the Hepatocyte System——Hz sulfate dissolved in the incubation medium was added to the hepatocyte system to a final concentration of 0.1 mM. After incubation for 15, 30, and 60 min, 1 ml of 40% zinc sulfate and 2 ml of saturated barium hydroxide were added to 1 ml of the incubation mixture with ^{15}N-Hz (1 μg/ml) as an internal standard. The resultant precipitate was sedimented by centrifugation, and an aliquot of the supernatant fluid was extracted twice with 20 ml of ethyl acetate. The combined extract was evaporated to dryness under a stream of nitrogen. Determination of Hz was performed by GC-MS as already reported.[11]

Evaluation of Toxicity to Hepatocytes——The incubation at 37 °C was started within 1 h after the final resuspension of the cell pellets. Hz or AcHz was added to the rotating round-bottomed flasks to make a final concentration of 1 mM, and the suspension was incubated for 1 h under an atmosphere of 95% O_2–5% CO_2. The solution was sampled at 0, 15, 30 and 60 min and cell damage was checked by means of the trypan blue exclusion test.[13] The cells were separated from the reaction medium and the intracellular K^+ was estimated by the method described by Baur *et al.*[14] A 400 μl polyethylene microcentrifuge tube was filled with 100 μl of 0.7 M perchloric acid (PCA) solution followed by 200 μl of 1-bromododecane on top of it. On top of the oil was placed a 100 μl aliquot of cell suspension. Centrifugation was carried out at 10000 rpm for 10—15 s. The K^+ in the PCA layer (cell portion) was analyzed by flame photometry (Shimadzu AA-640-12 flame photometer). GSH and oxidized glutathione (GSSG) analyses were carried out according to Watanabe *et al.*[15]

Measurements of Rat Hepatic Cytochrome P-450 Contents and the Difference Spectrum of Hz with the Cytochrome P-450——The total amount of cytochrome P-450 was assayed by using hepatocytes disrupted by freezing and thawing three times, by the method described in the previous paper.[6] The difference spectrum with cytochrome P-450 was measured with a Shimadzu MPS 2000 spectrometer.[11] The experiment was performed in the hepatocyte system described in the legend to Fig. 3.

Results and Discussion

Figure 1 shows the time course of Hz disappearance after the incubation of 0.1 mM Hz with hepatocytes isolated from intact, PB- or RMP-pretreated rats. After incubation for 60 min with hepatocytes from the untreated group, the Hz concentration had decreased to 34% of the initial value (from 98 to 33 nmol/ml), while the Hz levels after 60 min with hepatocytes from PB- and RMP-pretreated rats were 21% and 29% of the initial value, respectively (significantly different from the untreated group). The half-lives of Hz disappearance were calculated as 0.71 h for the control group, 0.47 h for the PB-pretreated group

and 0.60 h for the RMP-pretreated one. The significant decrease in the latter two cases can be reasonably explained in terms of the increase of the hepatic cytochrome P-450,[16] since the main metabolic pathway of Hz is cytochrome P-450 dependent oxidation.[10]

In the previous study *in vivo*, we observed that the elimination rate of Hz was significantly increased in PB- and RMP-pretreated rats after intravenous administration.[8] In order to clarify the metabolic course of Hz, the effects of metyrapone and piperonylbutoxide, potent inhibitors of cytochrome P-450, were investigated using isolated rat liver cells. As shown in Fig. 2A, the decrease of Hz in the hepatocytes obtained from the control group was retarded by piperonylbutoxide addition (0.1 mM), while metyrapone (1.0 mM) did not have a significant effect. The difference between the inhibitory effects of these treatments may result from a difference in the extents of hepatocyte uptake of the respective compounds or inhibition of a specific isozyme of cytochrome P-450 which participates in the Hz oxidation. Figure 2B shows the effect of metyrapone on Hz disappearance in heaptocytes isolated from PB-pretreated rats

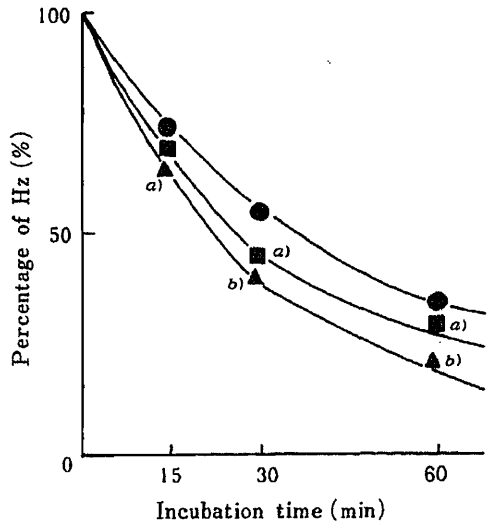

Fig. 1. Time Course of Hz Disappearance in Isolated Rat Hepatocytes

●, normal hepatocytes; ▲, PB-pretreated hepatocytes; ■, RMP-pretreated hepatocytes. The initial concentration of Hz was 0.1 mM. Each value represents the mean of 6—10 experiments. *a*) $p<0.05$; *b*) $p<0.01$ (significant difference from the percentage of Hz in the normal hepatocytes).

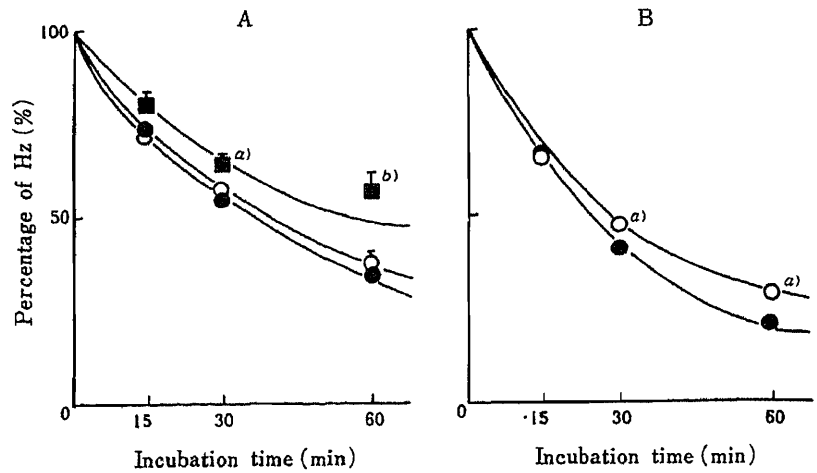

Fig. 2. Effects of Metabolic Inhibitors on Hz Disappearance in Isolated Rat Hepatocytes

A: normal hepatocytes. B: PB-pretreated hepatocytes. ●, Hz; ○, Hz+metyrapone (1 mM); ■, Hz+piperonyl butoxide (0.1 mM). The initial concentration of Hz was 0.1 mM. Each value represents the mean±S.E. of 5—10 experiments. *a*) $p<0.05$; *b*) $p<0.01$ (significant difference from the percentage of Hz without metabolic inhibitors).

in which the content of hepatic cytochrome P-450 (1.45 nmol/10^6 cells) was twice that of the control rats (0.72 nmol/10^6 cells). The Hz disappearance rate was significantly decreased by metyrapone addition in the hepatocytes isolated from PB-pretreated rats. Therefore, the results suggest an important role of PB-inducible cytochrome P-450 in Hz oxidation.

Therefore, we examined the difference spectrum of Hz with cytochrome P-450 in the hepatocytes. After immediate appearance of the type-II spectrum of Hz and the oxidized cytochrome P-450 (Fig. 3A), the formation of complexes between the metabolic intermediates of Hz and cytochrome P-450 was observed as a shift of the strong absorption maximum at 446 to 444 nm, which changed time-dependently from state B to state C in 8 min and thence to state E as shown in Fig. 3. Using rat liver microsomes, we preivously demonstrated the formation of diimide (HN=NH) as one of the metabolites of Hz.[11] Diimide affords an unstable complex with cytochrome P-450, showing an absorption maximum at 448 nm during the first 5 min. The peak at 448 nm was significantly inhibited by metyrapone. The observation of the peak at 446 nm in the present measurement supports the formation of diimide in the hepatocyte system. On the other hand, the peak at 444 nm given by the more stable complex (D and E) was not observed in the microsomal examination. Therefore, this complex is probably formed through a reaction catalyzed by an enzyme which is not present in the microsomal system but is in the hepatocytes. Furthermore, Hz radical (H_2N–$\dot{N}H$) has also been detected in rat liver microsomes as a reactive intermediate after incubation.[11] Later it was found that the same radical could also be formed by one electron oxidation catalyzed only by reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 reductase (fp_2) without cytochrome P-450 addition.[17] The significance of this is now under investigation.

In order to clarify the relationship between metabolism and cytotoxicity of Hz in the hepatocyte system, we carried out toxicological evaluation tests such as the trypan blue exclusion test and measurements of intracellular K^+ ion and glutathione levels. Figure 4 indicates the cell viability and the amount of K^+ retained in the hepatocytes isolated from PB- and RMP-pretreated rats after incubation with Hz at 37 °C for 15, 30 and 60 min. PB

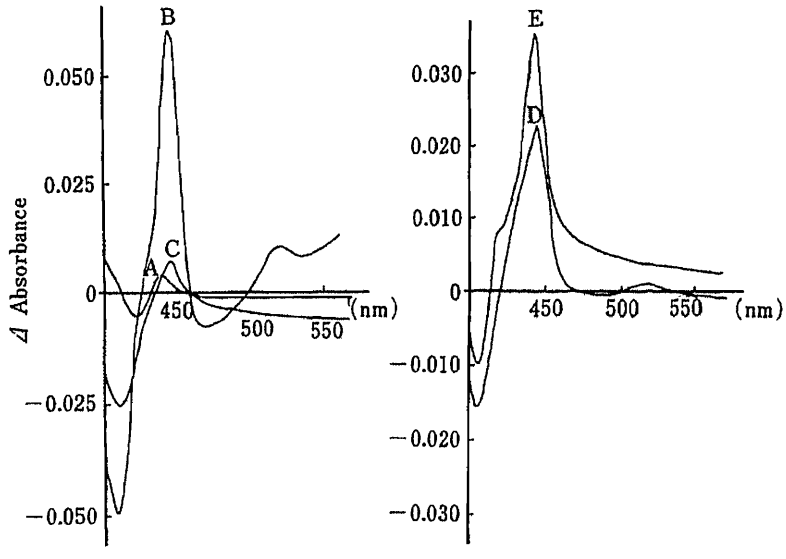

Fig. 3. Interaction of Hz with Cytochrome P-450 in Hepatocytes Isolated from PB-Pretreated Rats

A 4.8 ml aliquot of the suspension (3×10^6 cells/ml) was divided into two cuvettes. After recording a flat base-line, 0.1 ml of Hz solution was added at a concentration of 1.0 mM to the sample cuvette and an equal volume of buffer solution was added to the reference cuvette. The difference spectra were recorded at 37 °C just after shaking the sample solution.
A, 2 min; B, 5 min; C, 8 min; D, 15 min; E, 20 min.

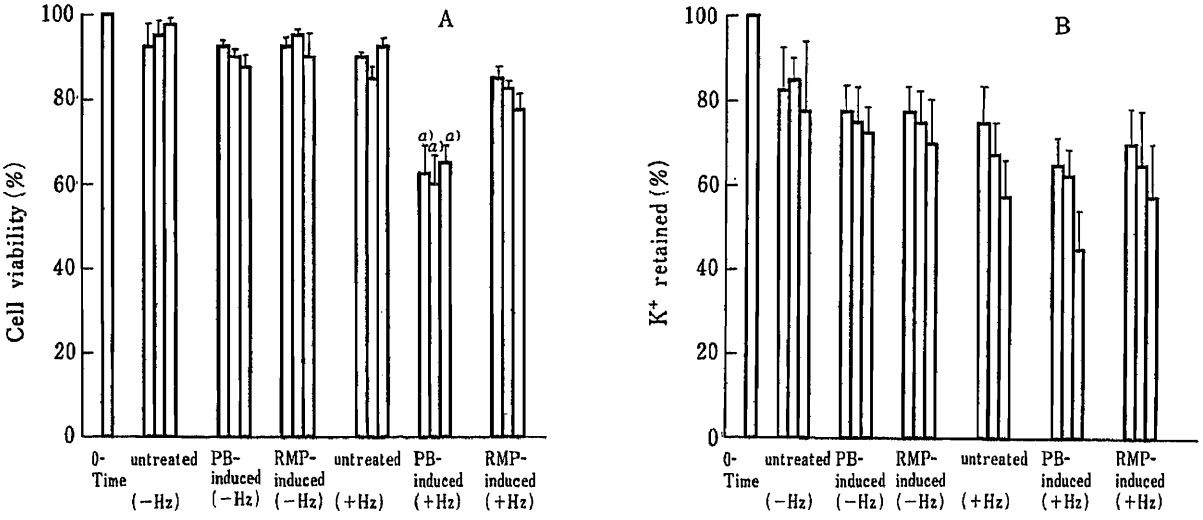

Fig. 4. Hz Toxicity Measured in Terms of Trypan Blue Viability Test (A) and Percentage of Intracellular K^+ (B)

A concentration of 1.0 mM Hz was employed. Each bar represents the mean percentage ± S.E. ($n=3$—4) with respect to the 0 time value at successive time points (15, 30 and 60 min) during the experiments. *a*) $p<0.05$ (significant difference from the value in the normal hepatocytes at each time point).

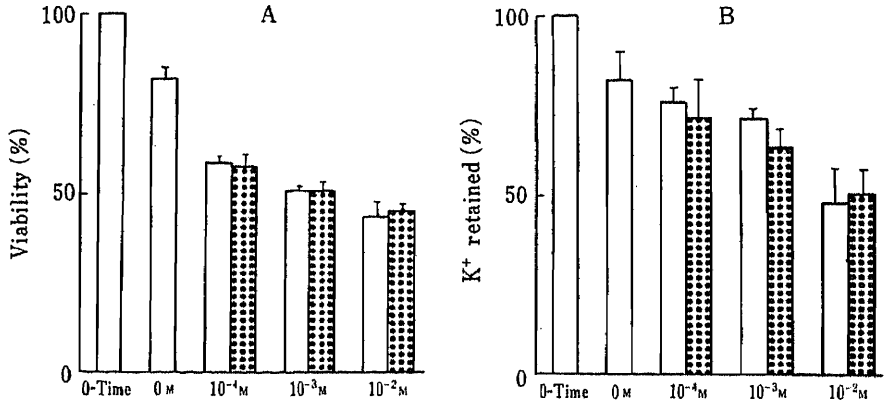

Fig. 5. Concentration Dependence of Toxicity of Hz (□) and Acetyl Hz (▨) Measured in Terms of the Trypan Blue Viability Test (A) and Percentage of Intracellular K^+ (B) in the Normal Hepatocytes after Incubation at 37 °C for 30 min

Each bar represents the mean percentage ± S.E. ($n=4$—5) with respect to the 0 time value without incubation in each hepatocyte system. The control experiment was performed by the addition of the incubation medium instead of the substrate solution.

pretreatment had a marked effect on the toxicity, while RMP pretreatment had only a slight effect. Therefore, the concentration dependence of the toxic effects of Hz was examined and compared with that of AcHz. Since the results showed that the cell viability and the amount of K^+ were reduced concentration-dependently by both Hz's (Fig. 5), cytotoxicity was demonstrated. However, no difference of cytotoxicity could be seen between Hz and AcHz.

The intracellular GSH content was significantly decreased not only with increasing Hz concentrations in the mixture but also with increasing incubation time with the hepatocytes isolated from both PB- and RMP-pretreated rats (Fig. 6). We observed no significant increase in the formation of GSSG, as shown in Table I. A similar tendency was reported by Watanabe

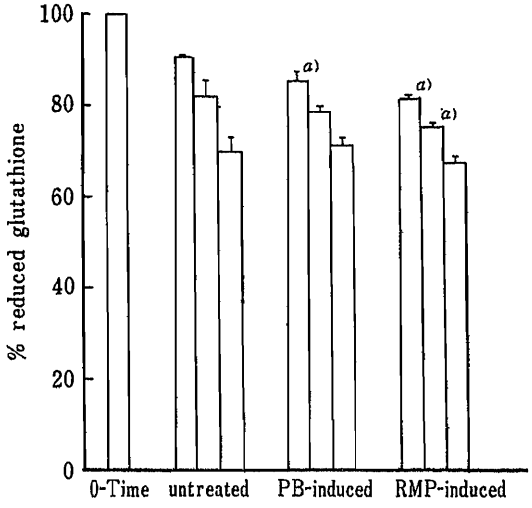

Fig. 6. Effect of Hz on GSH in Hepatocytes Isolated from PB- and RMP-Pretreated Rats

A concentration of 1.0 mM Hz was employed. Each bar represents the mean percentage ± S.E. ($n=5$—6) to the 0 time value without incubation at successive time points (15, 30 and 60 min) during incubation at 37 °C. *a*) $p<0.05$ (significant difference from the value obtained from the normal hepatocytes at each time point).

TABLE I. GSH and GSSG Levels in Isolated Rat Hepatocytes

	Glutathione (nmol/10^6 cells)		
	Total	Reduced	Oxidized
Control	123.92 ± 1.74	64.04 ± 4.52	26.89 ± 6.55
Hz (1 mM)	92.00 ± 10.58[a)]	46.69 ± 4.38[b)]	28.73 ± 4.30

Each value represents the mean ± S.E. of six experiments. *a*) $p<0.01$; *b*) $p<0.05$ (significant difference from the control value obtained from normal hepatocytes).

et al. in connection with the metabolism of 2-bromoethylaminonaphthoquinone in isolated rat hepatocytes.[15] Interestingly, AcHz did not have any effect on GSH content, as has already been reported by us.[9] The reason for the discrepancy in the effects of Hz and AcHz on GSH content remains to be clarified. In any case, the complex formation between the metabolic intermediate of Hz and cytochrome P-450 and the subsequent depletion of GSH may reflect Hz-induced hepatotoxicity, as reported in the case of SKF 525-A.[18]

In conclusion, Hz and AcHz are cytotoxic to isolated hepatocytes and the initial stage of the toxic response appears to involve damage to the cell membrane, as described by Siemens *et al.*[19] The experiments using the hepatocyte system probably mimic quite well the *in vivo* characteristics of Hz toxicity in the liver.

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Notes

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Dimethylthioxanthones Formed by Condensation of 2-Mercaptobenzoic Acid with *o*-, or *m*-Xylene in Sulfuric Acid

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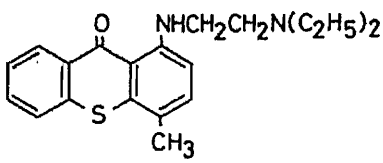
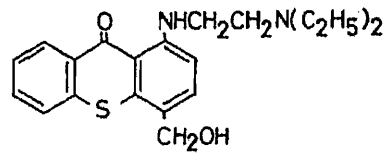
Various dimethyl-substituted thioxanthones were prepared by the condensation reaction of 2-mercaptobenzoic acid and *o*-, *m*-, or *p*-xylene in sulfuric acid. Some of them are novel compounds (**5**, **11**, **12**, and **13**). That is, from *o*-xylene, 1,2-dimethyl(**11**)-, 2,3-dimethyl(**12**)-, and 3,4-dimethylthioxanthones(**13**) were formed in yields of 9.3, 37.1, and 12.8%, respectively. From *m*-xylene and *p*-xylene, 2,4-dimethylthioxanthone (**4**) and 1,4-dimethylthioxanthone (**9**), respectively, were obtained by the same condensation reaction. The structures were confirmed on the basis of spectral investigation and comparison with authentic materials obtained by another synthetic route: the cyclization of phenylthiobenzoic acids.

Keywords——thioxanthone; thioxanthene; xylene; 2-mercaptobenzoic acid; 2-chlorobenzoic acid

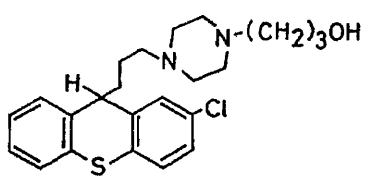
Some thioxanthene derivatives are used to treat various forms of mental disease. Kikuth and Gonnert[1] demonstrated that a series of synthetic thioxanthones were orally effective against schistosoma mansoni infection in white mice and monkeys. Of these thioxanthones, lucanthone and hycantone[2-5] (Fig. 1) were found to be effective both in experimental animal infections and in natural infections of humans. Recently, it has been shown that the phenothiazine nucleus may be replaced by thioxanthene without greatly altering the effects upon psychotic symptomatology (for example thiothixene and xanthiol).[6] In order to develop more effective thioxanthene derivatives as antipsychotic agents for the treatment of schizoph-



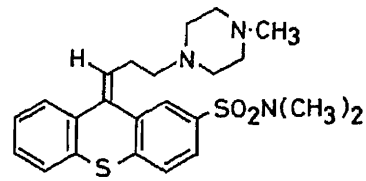
lucanthone



hycanthone



xanthiol



thiothixene

Fig. 1

renia, we have been studying the chemical and biochemical properties of thioxanthene derivatives.[7,8] In this paper, we describe the formation of various isomers of dimethylthioxanthones by the reaction of 2-mercaptobenzoic acid with *o*-, *m*-, or *p*-xylene in sulfuric acid. The structures of some of them were confirmed by chemical and physicochemical evidence and comparison with authentic materials obtained by another synthetic route, as shown in Charts 1 and 2.

It is well known that thioxanthone (**3**) is formed by the reaction of 2-mercaptobenzoic acid (**1**) with benzene in sulfuric acid under mild reaction conditions.[9] Prescott and Smiles[10] reported that the thioxanthone (**3**) was also formed by using 2,2′-dithiodibenzoic acid (**2**) in place of **1**.

With respect to the formation of dimethyl-substituted thioxanthones, Marsden and Smiles[11] reported that the reaction of **2** with *m*-xylene in sulfuric acid gave 1,3-dimethylthioxanthone (**5**) (mp 127 °C). On the other hand, Urberg and Kaiser[12] obtained 2,4-dimethylthioxanthone (**4**) (mp 142.5—143 °C) instead of **5** from the same reaction. They determined the structure of **4** on the basis of elemental analysis, and mass spectra (MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectra, and suggested that the lower melting point for the compound reported by Marsden and Smiles might have been due to contamination with **5** formed as a by-product. Our repetition of Marsden and Smiles' work gave a single product

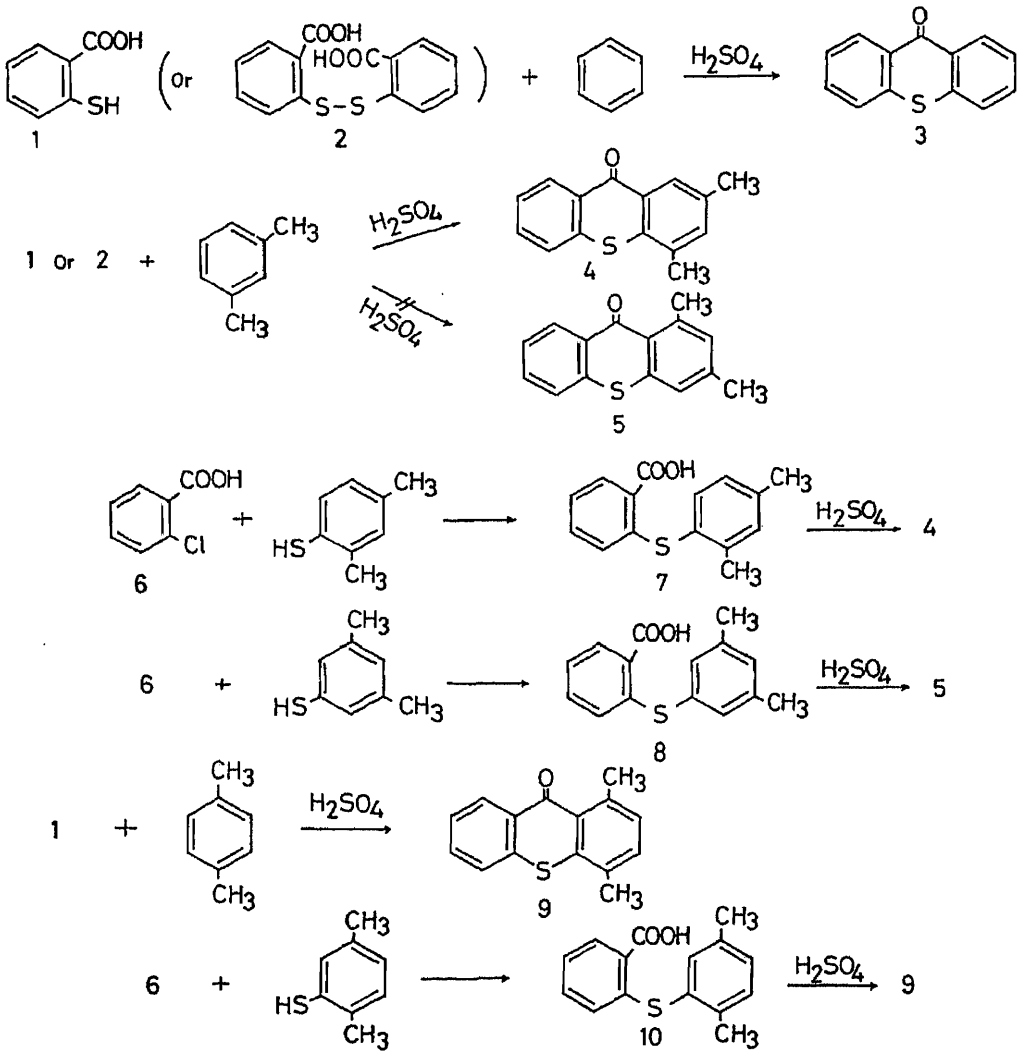


Chart 1

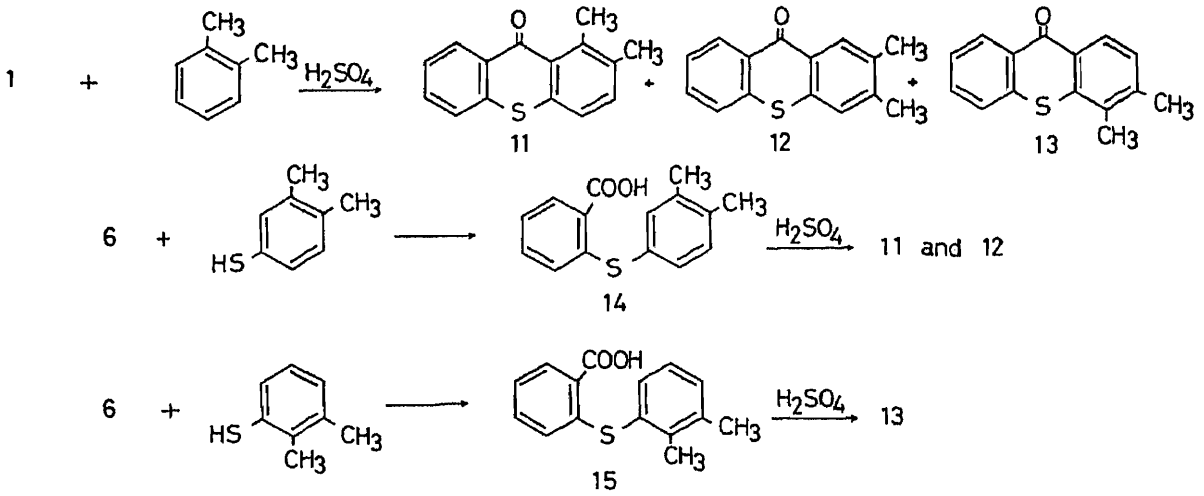


Chart 2

(mp 144—145 °C) whose ^{1}H-NMR spectrum supported the 2,4-dimethylthioxanthone structure (**4**). The structure of **4** was confirmed by comparison with authentic **4** prepared by cyclization of 2-(2,4-dimethylphenylthio)benzoic acid (**7**) obtained by means of the Ullmann reaction using 2-chlorobenzoic acid (**6**) and 2,4-dimethylbenzenethiol.[13] Our further investigation of Marsden's method using **2** showed that **4** was formed in a yield of 30%, and 70% of **2** was recovered. On the other hand, the condensation of **1** with *m*-xylene in sulfuric acid gave **4** in a yield of 83% under the same reaction conditions. That is, for the synthesis of **4**, it was found that the use of **1** was more favorable than that of **2**. The isomer of **4**, 1,3-dimethylthioxanthone (**5**) (mp 110—111 °C) was prepared from 2-(3,5-dimethylphenylthio)benzoic acid (**8**), which was formed from **6** and 3,5-dimethylbenzenethiol[13] in a manner similar to that described for the preparation of **4** *via* **7**. Similarly, the reaction of 2-mercaptobenzoic acid (**1**) and *o*- or *p*-xylene in sulfuric acid was carried out in order to obtain novel dimethyl-substituted thioxanthones related to **4**. Compound **9** was identical with the product alternatively prepared by cyclization of 2-(2,5-dimethylphenylthio)-benzoic acid (**10**) [obtained from **6** and 2,5-dimethylbenzenethiol][13] as shown in Chart 1.

Finally, the condensation of 2-mercaptobenzoic acid (**1**) with *o*-xylene in sulfuric acid gave three isomers of dimethyl-substituted thioxanthones; 1,2-dimethyl(**11**)-, 2,3-dimethyl(**12**)-, and 3,4-dimethylthioxanthone (**13**), as shown in Chart 2. These structures were confirmed on the basis of infrared (IR), ^{1}H-NMR, and mass spectral evidence. In addition, the identities of 1,2-dimethyl(**11**)- and 2,3-dimethylthioxanthone (**12**) were confirmed by comparison with authentic materials obtained by cyclization of 2-(3,4-dimethylphenylthio)benzoic acid (**14**), which was formed by the condensation of 2-chlorobenzoic acid (**6**) with 3,4-dimethylbenzenethiol as shown in Chart 2. Similarly, 3,4-dimethylthioxanthone (**13**) was identical with the product obtained from 2-(2,3-dimethylphenylthio)-benzoic acid (**15**) formed by the condensation of **6** with 2,3-dimethylbenzenethiol.

Experimental

Spectroscopy——The ^{1}H-NMR spectra and totally decoupled and off-resonance decoupled carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were measured in $CDCl_3$ solution in 5 mm tubes on a JEOL FX-200 spectrometer. Chemical shifts are relative to tetramethylsilane. The IR spectra were measured on a JASCO A-3 spectrometer. The electron impact (EI) mass spectra were obtained on a Hitachi RMU-7M mass spectrometer.

Chromatography——The thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} strips, 9.5 × 3.5 cm (0.25 mm thick) with $CHCl_3$ or ethyl acetate (AcOEt). The column chromatography was carried out on

Wakogel C-200 and Merck Aluminiumoxid 90.

Melting Points——The melting points were measured with a Yanagimoto micro-melting point apparatus and are uncorrected.

2,4-Dimethylthioxanthone (4)——a) A mixture of **7** (0.5 g) and conc. H_2SO_4 (2.5 ml) was heated in a boiling water bath for 30 min, and poured into ice-water (60 ml). The precipitates were collected and extracted with hot 5% $NaHCO_3$ solution. The insoluble product (0.2 g, 43%) was recrystallized from EtOH to give yellow needles, mp 144.5—145 °C. IR (KBr): 1630, 1585 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.43 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 7.28 (s, 1H, 3-CH), 7.32—7.64 (m, 3H, 5, 6, 7-CH), 8.28 (s, 1H, 1-CH), 8.55 (d, 1H, J=8 Hz, 8-CH). MS *m/z*: 240 (M^+). *Anal.* Calcd for $C_{15}H_{12}OS$: C, 74.97; H, 5.03. Found: C, 74.68; H, 4.82.

b) A mixture of **1** (1.0 g), conc. H_2SO_4 (10 ml) and *m*-xylene (3 ml) was stirred for 10 h; the reaction mixture was allowed to stand for 10 h and finally heated for 1 h in a boiling water bath. Water was poured over the surface of the reaction mixture and allowed to diffuse slowly into the mixture. The precipitates were collected and extracted with hot 5% $NaHCO_3$ solution. The insoluble product (1.3 g, 83%) was recrystallized from EtOH to give yellow needles, mp 144.5—145 °C, both alone and admixed with a sample obtained by method a). The IR and NMR spectra were identical with those of a sample obtained by method a).

c) A mixture of **2** (0.5 g), conc. H_2SO_4 (2.5 ml) and *m*-xylene (3 ml) was treated in a manner similar to that described in method b). The crude product (0.1 g, 13%) was recrystallized from EtOH to give yellow needles, mp 144—145 °C, both alone and admixed with a sample obtained by method a) or b).

2-(2,4-Dimethylphenylthio)benzoic Acid (7)——2-Chlorobenzoic acid (**6**) (2.5 g), 2,4-dimethylbenzenethiol (2.4 g) and a trace of copper powder were added to a solution of Na (0.74 g) in MeOH (20 ml). The MeOH was distilled off and the semisolid residue was heated in an oil bath at 100 °C. The temperature was raised to 230 °C during a period of 20 min with occasional stirring and held there for 15 min. The mixture was cooled, extracted with hot water and filtered. The filtrate was acidified with HCl. The precipitates (2.8 g, 68%) were collected and recrystallized from aqueous MeOH to give pale brown prisms, mp 231—234 °C. IR (KBr): 1670, 1600, 1580 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.30 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 6.61 (dd, 1H, J=8, 1 Hz), 7.00—7.28 (m, 4H, aromatic H), 7.44 (d, 1H), 8.11 (dd, 1H, J=8, 1 Hz, 6-CH). MS *m/z*: 258 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_2S$: C, 69.74; H, 5.46. Found: C, 69.50; H, 5.18.

2-(3,5-Dimethylphenylthio)benzoic Acid (8)——This compound was prepared from **6** (2.5 g) and 3,5-dimethylbenzenethiol (2.4 g) in a manner similar to that used for the preparation of **7**. The product (1.4 g, 34%) was recrystallized from aqueous MeOH to give colorless needles, mp 211—214 °C. IR (KBr): 1680, 1600, 1560 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.16 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 6.81 (dd, 1H, J=10, 1 Hz), 7.05 (s, 1H), 7.06—7.32 (m, 4H), 8.11 (dd, 1H, J=10, 1 Hz, 6-CH). MS *m/z*: 258 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_2S$: C, 69.74; H, 5.46. Found: C, 69.49; H, 5.25.

1,3-Dimethylthioxanthone (5)——This compound was prepared from **8** (0.5 g) and conc. H_2SO_4 (2.5 ml) in a manner similar to that described for the preparation of **4**. The product (0.2 g, 43%) was recrystallized from aqueous EtOH to give colorless needles, mp 110—111 °C. IR (KBr): 1630, 1600, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (s, 3H, CH_3), 2.86 (s, 3H, CH_3), 7.03 (s, 1H, 4-CH), 7.19 (s, 1H, 2-CH), 7.32—7.57 (m, 3H, 5, 6, 7-CH), 8.45 (d, 1H, J=10 Hz, 8-CH). MS *m/z*: 240 (M^+). *Anal.* Calcd for $C_{15}H_{12}OS$: C, 74.97; H, 5.03. Found: C, 74.67; H, 4.83.

2-(2,5-Dimethylphenylthio)benzoic Acid (10)——This compound was prepared from **6** (4 g) and 2,5-dimethylbenzenethiol (3.8 g) in a manner similar to that used for the preparation of **7**. The product (4.4 g, 67%) was recrystallized from aqueous MeOH to give colorless crystals, mp 171—174 °C. IR (KBr): 1670, 1580, 1550 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.31 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 6.62 (dd, 1H, J=10, 1 Hz), 7.04—7.32 (m, 4H, aromatic H), 7.41 (s, 1H), 8.15 (dd, 1H, J=10, 1 Hz, 6-CH). MS *m/z*: 258 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_2S$: C, 69.74; H, 5.46. Found: C, 69.36; H, 5.21.

2-(3,4-Dimethylphenylthio)benzoic Acid (14)——This compound was prepared from **6** (3 g) and 3,4-dimethylbenzenethiol (2.8 g) in a manner similar to that described for the preparation of **7**. The product (4 g, 81%) was recrystallized from aqueous MeOH to give pale brown plates, mp 222—224 °C. IR (KBr): 1678 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.28 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 6.74—7.28 (m, 6H, aromatic H), 8.11 (d, 1H, 6-CH). MS *m/z*: 258 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_2S$: C, 69.74; H, 5.46. Found: C, 69.53; H, 5.27.

2-(2,3-Dimethylphenylthio)benzoic Acid (15)——This compound was prepared from **6** (4 g) and 2,3-dimethylbenzenethiol (3.8 g) in a manner similar to that used for the preparation of **7**. The product (3.7 g, 56%) was recrystallized from aqueous MeOH to give pale brown needles, mp 243—246 °C. IR (KBr): 1665 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.32 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 6.56—7.46 (m, 6H, aromatic H), 8.12 (d, 1H, 6-CH). MS *m/z*: 258 (M^+). *Anal.* Calcd for $C_{15}H_{14}O_2S$: S, 69.74; H, 5.46. Found: C, 69.48; H, 5.19.

1,2-Dimethylthioxanthone (11), 2,3-Dimethylthioxanthone (12), and 3,4-Dimethylthioxanthone (13)——a) Preparation of **11**, **12**, and **13** by the Reaction of 2-Mercaptobenzoic Acid with *o*-Xylene: A mixture of **1** (2 g), conc. H_2SO_4 (6 ml) and *o*-xylene (7 g) was stirred for 1 h at room temperature, after which the reaction mixture was refluxed for 3 h and stirred for 5 h at room temperature. The resulting suspension was poured into ice-water (150 ml). The precipitates were collected and suspended in a saturated solution of $NaHCO_3$. The resulting suspension was extracted with $CHCl_3$, and the organic phase was dried (Na_2SO_4) and concentrated to give a greenish gray solid (2 g,

64%) containing **11**, **12**, and **13** (mp 125—160 °C).

The crude solid (2 g) was column-chromatographed over silica gel (100 g). Elution with *n*-hexane–AcOEt (5 : 1, v/v) afforded two fractions; the first fraction gave 1,2-dimethylthioxanthone (**11**) (160 mg) as colorless needles, and the second fraction contained a mixture (960 mg) of 2,3-dimethylthioxanthone (**12**) and 3,4-dimethylthioxanthone (**13**). This mixture was applied again to a column of alumina (100 g) with *n*-hexane–AcOEt (5 : 2, v/v) to afford **12** (640 mg) and **13** (220 mg) as pale yellow prisms.

Compound **11** was obtained as colorless plates, mp 103—104 °C alone and on admixture with an authentic sample obtained by method b). IR (KBr): 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.40 (s, 3H, CH_3), 2.75 (s, 3H, CH_3), 7.20—7.60 (m, 5H, aromatic H), 8.38 (d, 1H, J = 10 Hz, 8-CH). MS *m/z*: 240 (M^+). *Anal.* Calcd for $C_{15}H_{12}OS$: C, 74.97; H, 5.03. Found: C, 74.69; H, 4.81.

Compound **12** was obtained as pale yellow needles, mp 176—177 °C alone and on admixture with an authentic sample obtained by method b). IR (KBr): 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.36 (s, 6H, 2 × CH_3), 7.20—7.62 (m, 3H, aromatic H), 7.25 (s, 1H), 8.36 (s, 1H, 1-CH), 8.58 (d, 1H, J = 10 Hz, 8-CH). MS *m/z*: 240 (M^+). *Anal.* Calcd for $C_{15}H_{12}OS$: C, 74.97; H, 5.03. Found: C, 74.75; H, 4.83.

Compound **13** was obtained as pale brown needles, mp 196—198 °C alone and on admixture with an authentic sample obtained by method c). IR (KBr): 1625, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.44 (s, 6H, 2 × CH_3), 7.29 (d, 1H, J = 10 Hz, 2-CH), 7.34—7.58 (m, 2H, 6, 7-CH), 7.60 (d, 1H, J = 8 Hz, 5-CH), 8.42 (d, 1H, J = 10 Hz, 1-CH), 8.57 (d, 1H, J = 10 Hz, 8-CH). MS *m/z*: 240 (M^+). *Anal.* Calcd for $C_{15}H_{12}OS$: C, 74.97; H, 5.03. Found: C, 75.03; H, 5.21.

b) Preparation of **11** and **12** by Cyclization of **14**: A mixture of **14** (1 g) and conc. H_2SO_4 (5 ml) was heated in a boiling water bath for 30 min, and poured into ice-water (60 ml). The precipitates were collected and extracted with 5% $NaHCO_3$ solution. The alkaline suspension was extracted with $CHCl_3$ and the organic phase was dried (Na_2SO_4) and evaporated to give pale brown needles (0.38 g, 41%) consisting of **11** and **12**. The product was applied to a silica gel column with *n*-hexane–AcOEt (5 : 1, v/v). The first fraction gave 1,2-dimethylthioxanthone (**11**) (150 mg) as colorless plates, and the second fraction gave 2,3-dimethylthioxanthone (**12**) (180 mg) as pale yellow needles.

c) Preparation of **13** by Cyclization of **15**: A mixture of **15** (1 g) and conc. H_2SO_4 (5 ml) was heated in a boiling water bath for 30 min, and poured into ice-water (60 ml). The precipitates were collected and extracted with 5% $NaHCO_3$ solution. The alkaline suspension was extracted with $CHCl_3$ and the organic phase was dried (Na_2SO_4) and evaporated to give pale brown needles (0.42 g, 45%). Recrystallization from aqueous EtOH gave pale brown needles.

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Amino Acids and Peptides. XVI. Synthesis of N^G-Tosylarginyl Peptide Derivatives–Observation of Lactam Formation of Arginyl Residue[1,2]

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Z–Arg(Tos)–Pro–NHNHBoc (1) and Z–Arg(Tos)–Val–NHNHBoc (2) were prepared by the DCC, DCC–HOBt, DCC–DNp, mixed anhydride and DPPA methods. In each coupling reaction, lactam formation from the N^G-tosylarginyl residue was observed, although the extent of formation was different depending not only on the carboxyl activation method but also on the kind of amino component. Addition of HOBt suppressed the formation of acylurea but did not suppress the formation of the lactam in the synthesis of 2. Addition of DNp suppressed the formation of the lactam slightly although it did not improve the yield of the target peptides. In the mixed anhydride method, a fairly large amount of the lactam was obtained in the synthesis of Z–Arg(Tos)–Pro–NHNHBoc, and a urethan-type derivative, N^{α}-isobutyloxycarbonyl-Pro–NHNHBoc, was also formed. However, in the case of synthesis of Z–Arg(Tos)–Val–NHNHBoc, lactam formation was suppressed compared with other activation methods and a small amount of urethan-type derivative was obtained. In both cases, the DPPA method gave a fairly good yield of the target peptide with only a trace amount of lactam formation.

Keywords——N^G-tosylarginyl peptide; chemical synthesis; activation method; lactam formation; isolation; identification

It is well known that the earliest method of protection of the guanidino function of arginine was stimulated by the availability of nitroarginine.[3] However, the nitroguanidine group has sufficient nucleophilic character to react with intramolecular electrophilic centers, resulting in formation of a lactam, a derivative of 2-piperidone.[4] In fact, the lactam derived from Z–Arg(NO_2)–OH was isolated previously.[5] Although substitution with electron-withdrawing arylsulfonyl groups renders the guanidine less sensitive to nucleophiles such as hydrazine or ammonia, lactam formation is not still impeded in N^G-tosylarginine.[6] This paper describes syntheses of N^G-tosylarginyl peptides and isolation of the lactam derived from Z–Arg(Tos)–OH by activation of the carboxyl group.

In order to prepare active fragments of kininogen and angiotensinogen, Z–Lys(Z)–Arg(Tos)–Pro–NHNHBoc (3) and Z–Asp(OBzl)–Arg(Tos)–Val–NHNHBoc (4), respectively, were required. During the synthesis of the peptides described above, several methods were used to prepare N^G-tosylarginyl peptides. Z–Arg(Tos)–OH was coupled with H–Pro–NHNHBoc or H–Val–NHNHBoc to afford Z–Arg(Tos)–Pro–NHNHBoc (**1**) or Z–Arg(Tos)–Val–NHNHBoc (**2**), respectively. However, besides the desired peptides, the piperidone derivative from Z–Arg(Tos)–OH was obtained in every case, although the extent of its formation depended not only on the carboxyl activation method but also on the N-terminal amino acid residue of the N-component. In each coupling reaction, crude products were purified by silica gel column chromatography. The DCC method in the synthesis of

TABLE I. Yield of N^G-Tosylarginyl Peptide Derivatives and Lactam

Carboxyl component	Amino component	Activation method	Yield of products (%)[a]	
			Desired peptide	Lactam
Z-Arg(Tos)-OH	H-Pro-NHNHBoc	DCC	41.2	5.0
		DCC-HOBt	74.2	trace
		DCC-DNp	42.9	2.2
		Mixed anhydride	48.6	10.0
		DPPA	83.9	trace
	H-Val-NHNHBoc	DCC	38.6	trace
		DCC-HOBt	43.3	13.0
		DCC-DNP	47.2	trace
		Mixed anhydride	50.0	trace
		DPPA	82.0	trace

a) Yield was calculated on the basis of the amount of Z-Arg(Tos)-OH used.

Z-Arg(Tos)-Pro-NHNHBoc (**1**) and the DCC-HOBt method in the synthesis of Z-Arg(Tos)-Val-NHNHBoc (**2**) gave fairly large amounts of the lactam in a crystalline form. In the latter case, addition of HOBt did not suppress the formation of the lactam but increased it slightly. The DCC-DNp[7] method, where Z-Arg(Tos)-OH was activated before combining with the N-component, gave a small amount of lactam in a crystalline form besides the desired peptide. In the mixed anhydride procedure to prepare **1**, not only the lactam from Z-Arg(Tos)-OH but also the urethan-type product, N^{α}-isobutyloxycarbonyl-Pro-NHNHBoc,[8,9] was obtained, besides the desired product. On the other hand, in the coupling of Z-Arg(Tos)-OH with H-Val-NHNHBoc by the mixed anhydride procedure, formation of the lactam and urethan-type products was only slight. The DPPA method was also used for the preparation of **1** and **2**. It was found that the coupling of Z-Arg(Tos)-OH with H-Pro-NHNHBoc or H-Val-NHNHBoc was achieved most effectively by the DPPA method. These results are summarized in Table I.

After removal of the Z group of **1** or **2** by catalytic hydrogenation, Z-Lys(Z)-ONp or Z-Asp(OBzl)-ONp was coupled, respectively, and the crude product was purified by silica gel column chromatography to give Z-Lys(Z)-Arg(Tos)-Pro-NHNHBoc (**3**) or Z-Asp(OBzl)-Arg(Tos)-Val-NHNHBoc (**4**) in a pure form to construct the active fragment of kininogen or angiotensinogen.

In the synthesis of N^G-tosylarginyl peptides, it must be considered that lactam formation from Z-Arg(Tos)-OH is inevitable during peptide bond formation by activation of the carboxyl group of Z-Arg(Tos)-OH. The extent of its formation depends not only on the carboxyl activation method employed but also on the amino acid residue of the N-component. The lactam is fairly readily crystallizable from MeOH, and separation of the lactam derivative from the desired peptide by recrystallization is difficult.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6N HCl, 110°C, 18 h) were determined with an amino acid analyzer (K-101 AS; Kyowa Seimitsu Co., Ltd.). For column chromatography, Kieselgel 60 (70—230 mesh, Merck) was used. On thin-layer chromatography (Kieselgel G, Merck), Rf^1, Rf^2 and Rf^3 values refer to the systems of $CHCl_3$ and MeOH (98 : 2), AcOEt and EtOH (4 : 1) and $CHCl_3$, MeOH and AcOH (90 : 8 : 2), respectively.

Z-Arg(Tos)-Pro-NHNHBoc (1)——1) DCC Method: Z-Arg(Tos)-OH (2.5 g) and H-Pro-NHNHBoc (pre-

pared from 2.0 g of Z-Pro-NHNHBoc[10] by catalytic hydrogenation) were dissolved in DMF (30 ml), and the solution was cooled with ice-salt. DCC (1.2 g) was added to the cold solution, and the reaction mixture was stirred at room temperature for 24 h. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Addition of ether and petroleum ether gave a solid mass. The crude product in $CHCl_3$ (5 ml) was applied to a column of silica gel (3.5 × 10 cm), which was eluted first with $CHCl_3$ (300 ml) and then with 2% MeOH in $CHCl_3$. 1-Tosylguanyl-3-benzyloxycarbonylaminopiperidone-2 (lactam) was eluted with the latter solvent (1—150 ml), and the product was recrystallized from MeOH, mp 157—158 °C, $[\alpha]_D^{25}$ −19.7° (c=0.9, MeOH), Rf^1 0.73. *Anal.* Calcd for $C_{21}H_{24}N_4O_5S$: C, 56.8; H, 5.44; N, 12.6. Found: C, 56.7; H, 5.35; N, 12.6. Further elution with the same solvent (200—450 ml) gave the desired dipeptide, which was recrystallized from AcOEt and petroleum ether; sintering at 105 °C and melting at 120 °C, $[\alpha]_D^{25}$ −61.4° (c=0.6, MeOH), Rf^1 0.05, Rf^2 0.74. *Anal.* Calcd for $C_{31}H_{43}N_7O_8S$: C, 55.3; H, 6.43; N, 14.6. Found: C, 55.6; H, 6.73; N, 14.1.

2) DCC–HOBt Method: Z-Arg(Tos)-OH (2.5 g), H-Pro-NHNHBoc (prepared from 2.0 g of Z-Pro-NHNHBoc) and HOBt (0.73 g) were dissolved in DMF (30 ml) and the solution was cooled with ice-salt. DCC (1.2 g) was added to the above cold solution. The reaction mixture was stirred at room temperature for 24 h. Isolation and purification of the products were carried out in the same way as described above. With $CHCl_3$, a trace amount of the lactam was obtained. With 1% MeOH in $CHCl_3$ (20—300 ml), *N*-[Z-Arg(Tos)]-urea was obtained, yield 0.1 g (2.7%), Rf^2 0.85, Rf^3 0.90. Further elution with the same solvent (500—1000 ml) and 2% MeOH in $CHCl_3$ (1—1000 ml) gave the desired peptide, mp 120 °C, Rf^1 0.05, Rf^2 0.75.

3) DCC–DNp Method: Z-Arg(Tos)-OH (2.4 g) and 2,4-dinitrophenol (1.0 g) were dissolved in DMF (30 ml) and the solution was cooled with ice-salt. DCC (1.2 g) was added to the above cold solution and the reaction mixture was stirred at room temperature for 3 h. After removal of the urea derivative, the filtrate was added to a cold solution of H-Pro-NHNHBoc (prepared from 2.0 g of Z-Pro-NHNHBoc) in DMF (20 ml). After 48 h, the crude products were obtained and purified in the same way as described above. Elution with 2% MeOH in $CHCl_3$ (25—175 ml) gave the lactam, which was recrystallized from MeOH, mp 156—158 °C, Rf^1 0.73. Further elution with the same solvent (200—450 ml) gave the desired peptide, sintering at 103 °C and melting at 118 °C, Rf^1 0.05, Rf^2 0.73.

4) Mixed Anhydride Method: A mixed anhydride (prepared from 2.6 g of Z-Arg(Tos)-OH, 0.6 ml of *N*-methylmorpholine and 0.72 ml of isobutyl chloroformate) in DMF (30 ml) was added to a solution of H-Pro-NHNHBoc (prepared from 2.0 g of Z-Pro-NHNHBoc) in DMF (20 ml). The reaction mixture was stirred at room temperature overnight. Isolation and purification of the products were performed in the same way as described above. Elution with 2% MeOH in $CHCl_3$ (25—200 ml) gave the lactam, which was recrystallized from MeOH, mp 157—158 °C, Rf^1 0.74. Further elution with the same solvent (200—350 ml) gave N^{α}-isobutyloxycarbonyl-Pro-NHNHBoc, yield 0.25 g (14.3%), mp 58—60 °C, $[\alpha]_D^{25}$ −68.8° (c=0.8, MeOH), Rf^1 0.48. *Anal.* Calcd for $C_{15}H_{27}N_3O_5$: C, 54.7; H, 8.26; N, 12.8. Found: C, 55.0; H, 8.00; N, 12.4. Further elution with the same solvent (360—660 ml) gave the desired product, mp 115—120 °C, Rf^1 0.05, Rf^2 0.73.

5) DPPA Method: Z-Arg(Tos)-OH (2.5 g) and H-Pro-NHNHBoc (prepared from 2.0 g of Z-Pro-NHNHBoc) were dissolved in DMF (30 ml) and the solution was cooled with ice-salt. DPPA (1.64 g), followed by Et_3N (1.67 ml), was added and the reaction mixture was stirred at 4 °C for 24 h. Isolation and purification of the products were performed in the same way as described above. Elution with 1% MeOH in $CHCl_3$ (250—700 ml) gave trace amounts of the lactam. Elution with 2% MeOH in $CHCl_3$ (50—1500 ml) yielded the desired peptide, mp 118 °C, Rf^1 0.05, Rf^2 0.75.

Z-Arg(Tos)-Val-NHNHBoc (2)——1) DCC Method: Z-Arg(Tos)-OH (3.2 g) and H-Val-NHNHBoc (prepared from 2.5 g of Z-Val-NHNHBoc[11] were dissolved in DMF (30 ml) and the solution was cooled with ice-salt. DCC (1.7 g) was added, and the reaction mixture was stirred at room temperature for 24 h. Isolation and purification of the products were carried out in the same way as described above. Elution with 1% MeOH in $CHCl_3$ (1—250 ml) gave a trace amount of the lactam. Further elution with the same solvent (270—750 ml) gave *N*-[Z-Arg(Tos)]-urea, yield 1.58 g (34.2%), mp 105—108 °C, $[\alpha]_D^{25}$ +14.4° (x=1.1, MeOH), Rf^2 0.85, Rf^3 0.90. *Anal.* Calcd for $C_{34}H_{48}N_6O_6S$: C, 61.1; H, 7.24; N, 12.6. Found: C, 61.0; H, 7.20; N, 12.4. Elution with 2% MeOH in $CHCl_3$ (50—750 ml) yielded the desired peptide, mp 114—120 °C, $[\alpha]_D^{25}$ −30.2° (c=0.6, MeOH), Rf^1 0.05, Rf^2 0.75. *Anal.* Calcd for $C_{31}H_{45}N_7O_8S$: C, 55.1; H, 6.71; N, 14.5. Found: C, 55.5; H, 7.00; N, 14.0.

2) DCC–HOBt Method: Z-Arg(Tos)-OH (6.4 g), H-Val-NHNHBoc (prepared from 5.0 g of Z-Val-NHNHBoc) and HOBt (1.85 g) were dissolved in DMF (50 ml) and the solution was cooled with ice-salt. DCC (3.4 g) was added, and the reaction mixture was stirred at room temperature for 24 h. Crude products were purified by silica gel column chromatography in the same manner as described above. Elution with $CHCl_3$ (350 ml) gave the lactam, mp 157—158 °C, Rf^1 0.74. Elution with 2% MeOH in $CHCl_3$ (370—970 ml) gave the desired peptide, and the product was recrystallized from AcOEt and petroleum ether, Rf^1 0.05, Rf^2 0.74.

3) DCC–DNp Method: Z-Arg(Tos)-OH (3.2 g) and 2,4-dinitrophenol (1.31 g) were dissolved in DMF (20 ml) and the solution was cooled with ice-salt. DCC (1.57 g) was added to the above cold solution. The reaction mixture was stirred at room temperature for 3 h. This solution was combined with H-Val-NHNHBoc (prepared from 2.5 g of Z-Val-NHNHBoc) in DMF (10 ml). After 48 h at room temperature, the products were isolated in the same way as

described above. Elution with $CHCl_3$ (250—700 ml) gave a trace amount of the lactam, Rf^1 0.73. Elution with 1% MeOH in $CHCl_3$ (1—700 ml) yielded a small amount of acylurea, Rf^2 0.84. Elution with 2% MeOH in $CHCl_3$ (200—1000 ml) gave the desired peptide, mp 110—118 °C, Rf^1 0.05.

4) Mixed Anhydride Method: Mixed anhydride (prepared from 8.3 g of Z-Arg(Tos)-OH, 2.3 ml of isobutyl chloroformate and 1.95 ml of *N*-methylmorpholine) in DMF (50 ml) was added to a solution of H-Val-NHNHBoc (prepared from 6.5 g of Z-Val-NHNHBoc). This reaction mixture was stirred at 0 °C overnight. The crude products were purified in the same way as described above. Elution with $CHCl_3$ (400 ml) gave a trace amount of the lactam, Rf^1 0.74. Elution with 2% MeOH in $CHCl_3$ (1—200 ml) gave a trace amount of the urethan-type product, Rf^1 0.60, followed (500—1300 ml) by the desired dipeptide derivative, mp 118—123 °C, Rf^1 0.05, Rf^2 0.75.

5) DPPA Method: Z-Arg(Tos)-OH (3.2 g) and H-Val-NHNHBoc (prepared from 2.5 g of Z-Val-NHNHBoc) were dissolved in DMF (25 ml) and the solution was cooled with ice-salt. DPPA (2.09 g), followed by Et_3N (2.12 ml), was added to the above solution. The reaction mixture was stirred in a cold room for 24 h. Elution with 1% MeOH in $CHCl_3$ (1—1000 ml) gave a trace amount of the lactam. Elution with 2% MeOH in $CHCl_3$ (200—1500 ml) yielded the desired peptide, mp 113—121 °C, Rf^1 0.05.

Z-Lys(Z)-Arg(Tos)-Pro-NHNHBoc (3)——Z-Lys(Z)-ONp (1.2 g) and H-Arg(Tos)-Pro-NHNHBoc (prepared from 1.5 g of Z-Arg(Tos)-Pro-NHNHBoc by catalytic hydrogenation) were dissolved in DMF (30 ml) containing triethylamine (0.3 ml). This reaction mixture was stirred at room temperature for 24 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The residue was purified by silica gel column (3.5 × 10 cm) chromatography. The column was eluted with $CHCl_3$ (300 ml), 1% MeOH in $CHCl_3$ (300 ml) and then 2% MeOH in AcOEt (400 ml). From the eluate with 2% MeOH in AcOEt, the desired peptide was obtained in a yield of 1.3 g (63%) as an amorphous powder, $[\alpha]_D^{25}$ −83.6° (c=0.6, MeOH), Rf^1 0.05, Rf^2 0.70. *Anal.* Calcd for $C_{42}H_{56}N_8O_{11}S \cdot 3.5H_2O$: C, 53.4; H, 6.72; N, 11.9. Found: C, 53.2; H, 6.47; N, 12.4. Amino acid ratios in an acid hydrolysate: Lys 1.00; Arg 0.85; Pro 1.05 (average recovery 81%).

Z-Asp(OBzl)-Arg(Tos)-Val-NHNHBoc (4)——Z-Asp(OBzl)-ONp (2.1 g) and H-Arg(Tos)-Val-NHNHBoc (prepared from 3.0 g of Z-Arg(Tos)-Val-NHNHBoc) were dissolved in DMF (30 ml) containing triethylamine (0.61 ml). The reaction mixture was stirred at room temperature for 24 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The residue was applied to a silica gel column (3.5 × 14 cm), which was eluted with $CHCl_3$ (300 ml), and 2% MeOH in $CHCl_3$. The latter eluate (300—700 ml) contained the desired peptide, which was recrystallized from AcOEt and petroleum ether, yield 1.8 g (43.7%), mp 115—120 °C, $[\alpha]_D^{25}$ −25.8° (c=0.2, MeOH), Rf^1 0.05, Rf^2 0.60. *Anal.* Calcd for $C_{45}H_{61}N_9O_{11}S \cdot H_2O$: C, 56.7; H, 6.66; N, 13.2. Found: C, 56.7; H, 6.75; N, 13.0. Amino acid ratios in an acid hydrolysate: Asp 1.00, Arg 0.9; Val 0.9 (average recovery 75%).

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Microbial Reduction of Naphthoxypropionic Acids

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Racemic 2-(1-naphthoxy)propionic acid ((±)-**1**) and 2-(2-naphthoxy)propionic acid ((±)-**4**) were subjected to microbial reduction and simultaneous resolution with *Glomerella cingulata* (*Malus* and *Prunus* strains), *Gloeosporium olivarum* (*G. olivarum*) and *Gloeosporium laeticolor* (*G. laeticolor*), yielding (*S*)-(+)-2-(1-naphthoxy)propanol ((*S*)-**2**) and (*R*)-(+)-2-(2-naphthoxy)-propanol ((*R*)-**5**), and leaving (*R*)-(−)-2-(1-naphthoxy)propionic acid ((*R*)-**1**) and (*S*)-(−)-2-(2-naphthoxy)propionic acid ((*S*)-**4**), respectively. The optical purity of (*S*)-(+)-2-(1-naphthoxy)-propanol ((*S*)-**2**) reached 92% and over 99% ee when fermented with *G. olivarum* and *Glomerella cingulata* (*Malus* strain), respectively.

Keywords——herbicide; microbial reduction; microbial resolution; 2-(1-naphthoxy)propanol; 2-(2-naphthoxy)propanol; 2-(1-naphthoxy)propionic acid; 2-(2-naphthoxy)propionic acid

2-(1-Naphthoxy)propionic acid *N,N*-diethylamide and 2-(2-naphthoxy)propionic acid anilide are well-known herbicides (commercial names Devrinol or Napropamide, and Naproanilide). Chan *et al.* reported[1] that the (*R*)-(−)-isomer of the former compound showed eightfold higher herbicidal activity than the (*S*)-(+)-isomer. Igarashi *et al.* reported[2] that only the (+)-isomer of the latter herbicide was active, while the (−)-isomer was almost inactive. Most herbicides, including Napropamide and Naproanilide, are currently used as their racemates only because the preparation of the active configurational form is tedious and uneconomical, even though the use of the active form is highly desirable in view of the residual toxicity of the inactive form of herbicides.

As part of a series of studies on the microbial transformation of 2-substituted propionic acids,[3] we report in this paper the microbial reduction, and simultaneous resolution, of 2-(1-naphthoxy)propionic acid ((±)-**1**) and 2-(2-naphthoxy)propionic acid ((±)-**4**). The racemic compound ((±)-**1**) was converted to chiral (*S*)-(+)-2-(1-naphthoxy)propanol ((*S*)-**2**) leaving (*R*)-(−)-2-(1-naphthoxy)propionic acid ((*R*)-**1**) in the culture broth, by the microbial action of the molds *Glomerella cingulata* (*Malus* and *Prunus* strains), *G. olivarum* and/or *G. laeticolor*. The ratio of the products (*R*)-**1** to (*S*)-**2** depended on the kind of strain used. It is noteworthy that the optical purity of the product ((*S*)-**2**) reached 92% ee with *G. olivarum*, and over 99% ee with *Glomerella cingulata* (*Malus* strain). It is very interesting, though the reason remains unknown, that the microbial action gave the reverse result in terms of the configuration of the product from (±)-**4** as compared with (±)-**1**, *i.e.*, (±)-**4** gave not the (*S*)- but the (*R*)-isomer ((*R*)-**5**) of 2-(2-naphthoxy)propanol as the reduction product, and left not the (*R*)-(+)- but the (*S*)-(−)-isomer) (*S*)-**4**) of 2-(2-napthoxy)propionic acid, after fermentation with *Glomerella cingulata* (*Malus* strain) or *G. olivarum*. The additive chemical yields of (*R*)-**1** and (*S*)-**2**, and also those of (*S*)-**4** and (*R*)-**5** were best, exceeding 80%, with *G. olivarum*. The reason for the low yields, below 60%, with the other molds may be metabolic degradation to other compounds.

The absolute configurations of the (*R*)-(−)-2-(1-naphthoxy)propionic acid ((*R*)-**1**) and

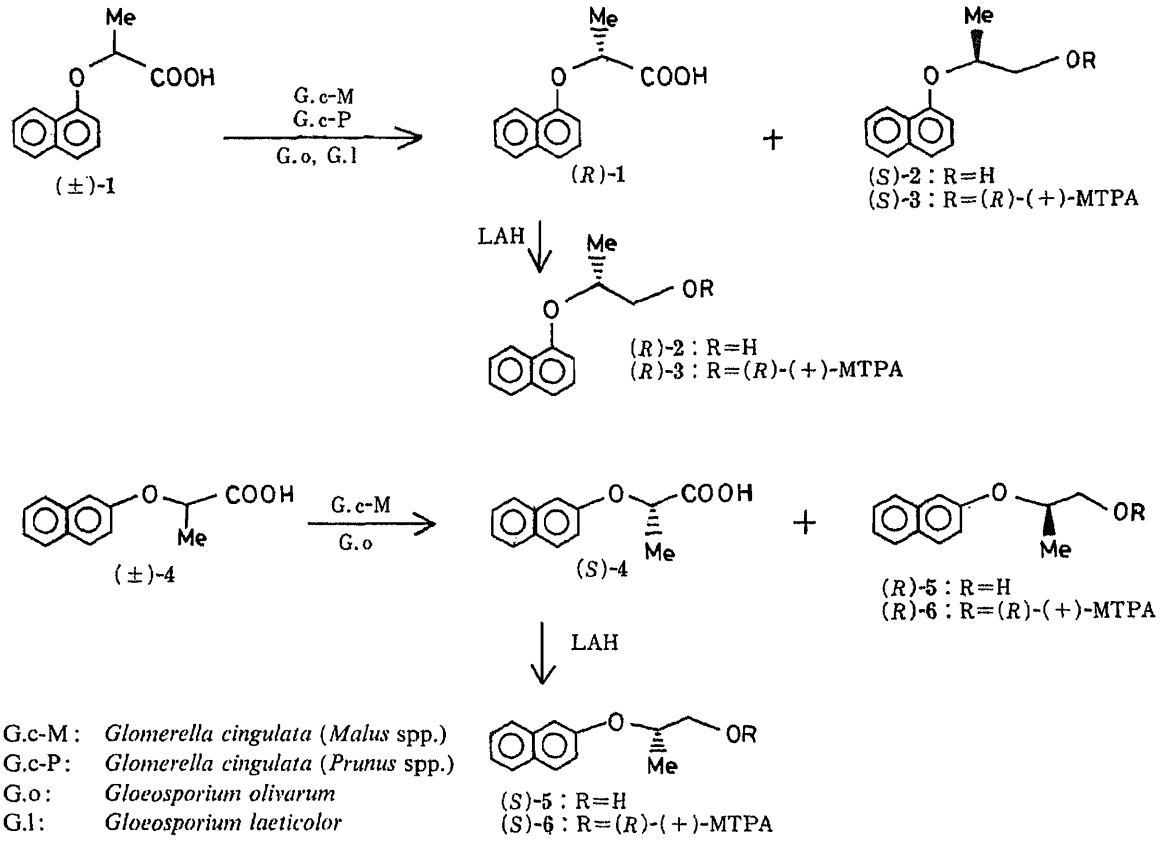

Chart 1

TABLE I. Asymmetric Reduction and Resolution of 2-(1-Naphthoxy)propionic Acid and 2-(2-Naphthoxy)propionic Acid

Substrate	Microorganism	Incubation (d)	Products	Yield (%)	$[\alpha]_D^{20}$ [a]	Optical purity
(±)-1	*G. cingulata* (*Malus* spp.)	24	(*R*)-1	43	−26.3 (C)	36[b]
			(*S*)-2	9	+19.4 (E)	>99[c]
	G. cingulata (*Prunus* spp.)	12	(*R*)-1	50	−8.7 (A)	18[b]
			(*S*)-2	15	+11.3 (E)	58[c]
	G. laeticolor	12	(*R*)-1	10	−28.0 (A)	59[b]
			(*S*)-2	47	+16.5 (E)	85[c]
	G. olivarum	15	(*R*)-1	42	−30.0 (C)	41[b]
			(*S*)-2	40	+17.7 (E)	92[c]
(±)-4	*G. cingulata* (*Malus* spp.)	26	(*S*)-4	19	−10.7 (E)	13[b]
			(*R*)-5	44	+7.9 (C)	20[c]
	G. olivarum	15	(*S*)-4	44	−9.3 (E)	7[b]
			(*R*)-5	36	+6.0 (C)	15[c]

a) Solvents: A, acetone; C, $CHCl_3$; E, EtOH. Concentration: $c=2.5$. *b*) Determined by the (*R*)-(+)-MTPA ester method. *c*) Determined by the (*R*)-(+)-MTPA ester method with the corresponding alcohol derived by reduction with $LiAlH_4$.

(*S*)-(−)-2-(2-naphthoxy)propionic acid ((*S*)-**4**) were determined by comparison of the values of specific rotation with those given in the literature.[4] These residual acids ((*R*)-**1** and (*S*)-**4**) were reduced to the respective (*R*)-2-(1-naphthoxy)propanol ((*R*)-**2**) and (*S*)-2-(2-naphthoxy)propanol ((*S*)-**5**) by the action of lithium aluminum hydride (LAH). The optical

purities of these alcohols ((*R*)-**2** and (*S*)-**5**), and hence those of the residual acids ((*R*)-**1** and (*S*)-**4**), were determined by deriving them into the (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA)[5] esters ((*R*)-**3** and (*S*)-**6**), and measuring the relative intensities of the methoxy 100 MHz proton nuclear magnetic resonance (^{1}H-NMR) signals of the (*R*,*R*)- and (*R*,*S*)-diastereomers in the presence of Sievers' lanthanide shift reagent, Eu(fod)$_3$.

The absolute configurations of 2-(1-naphthoxy)propanol ((*S*)-**2**) and 2-(2-naphthoxy)propanol ((*R*)-**5**) were determined by comparing their values of specific rotation with those of the alcohols ((*R*)-**2** and (*S*)-**5**). These alcohols ((*S*)-**2** and (*R*)-**5**) were further derived into their (*R*)-(+)-MTPA esters ((*S*)-**3** and (*R*)-**6**) and their optical purities were determined by the method described above. The incubation times, yields, values of specific rotation and optical purities of the fermentation products obtained with various mold strains are shown in Table I.

Experimental

Infrared (IR) spectra were determined on a Hitachi model 215 spectrometer. ^{1}H-NMR spectra were recorded at 100 MHz with tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer. Optical rotations were measured on a JASCO DIP-101 automatic polarimeter with a cell of 10 cm light path length. Gas-liquid chromatographic (GLC) analyses were performed on Shimadzu GC-4CM PF instrument with a flame ionization detector and a glass column (2 m × 3 mm) packed with 2% OV-1 on Chromosorb W, at a nitrogen pressure of 1 kg/cm^2. The mass spectra (MS) were obtained on a JEOL JMS-D 300 instrument.

Preparation of (*R*)-(+)-MTPA Ester ((±)-3) of Racemic 2-(1-Naphthoxy)propanol ((±)-2)——Pyridine (0.3 ml) was added to a mixture of the alcohol ((±)-**2**) (20 mg) and (*R*)-(+)-MTPA chloride (35 mg), and the mixture was stirred for 48 h at room temperature. After addition of water, the reaction mixture was extracted with ether, and the extract was washed with a small amount of saturated aq. NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to give an oil, which was subjected to preparative thin-layer chromatography (TLC) on silica gel (20 × 20 cm), with $CHCl_3$ as the eluting solvent, to provide pure (*R*)-(+)-MTPA ester ((±)-**3**) as an oil; yield 27 mg. MS *m/z*: 418 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.44 (3H, d, J=5.9 Hz, CH_3), 3.51 (3H, s, OMe), 4.66 (3H, m, CH_2 and CH), 6.78—8.15 (12H, m, ArH). ^{1}H-NMR (10 mg in $CDCl_3$ in the presence of 26.7 mg of Eu(fod)$_3$) δ: (*R*)-OMe 6.00 and (*S*)-OMe 6.46.

Preparation of (*R*)-(+)-MTPA Ester ((±)-6) of Racemic 2-(2-Naphthoxy)propanol ((±)-5)——The (*R*)-(+)-MTPA ester ((±)-**6**) was prepared from (±)-**5** (20 mg) in the same way as in the case of the preparation of the (±)-**3** from (±)-**2**. The pure ester ((±)-**6**) was obtained as an oil; yield 21 mg. MS *m/z*: 418 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.40 (3H, d, J=6.1 Hz, CH_3), 3.51 (3H, s, OMe), 4.54 (3H, m, CH_2 and CH), 7.02—7.77 (12H, m, ArH). ^{1}H-NMR (9 mg in $CDCl_3$ in the presence of 36.8 mg of Eu(fod)$_3$ δ: (*S*)-OMe 6.21 and (*R*)-OMe 6.29.

Microbial Reduction and Resolution of 2-(1-Naphthoxy)propionic Acid ((±)-1) and 2-(2-Naphthoxy)propionic Acid ((±)-4)——a) General Fermentation Procedures: Fermentation in a jar fermenter was not applicable, because the air-bubbling into the culture solution which was necessary for the growth of the molds was found to suppress the microbial reduction. Thus the stationary culture method was adopted. The cultivation period was adjusted as necessary for growing the mold.

A substrate (3 g) was added to the culture broth containing peptone (60 g), KH_2PO_4 (6 g), $MgSO_4 \cdot 7H_2O$ (2.4 g), sucrose (300 g) and water (6 l), and the whole mixture was kept in 20 Roux bottles after ajustment of the pH to 5.5. The Roux bottles were stoppered with cotton, autoclaved, cooled, and inoculated with a microorganism. Culture was carried out at 27 °C for the period designated in Table I. Longer incubation periods resulted in a decrease of the optical purity of the produced alcohols, though the yield increased. After removal of the mycelium by filtration, the culture filtrate was alkalized to pH 10, and shaken with ether (2 l). The ethereal extract was washed with water and dried over anhydrous Na_2SO_4. The crude product obtained after evaporation of the solvent was purified by column chromatography on silica gel, using $CHCl_3$ as the eluting solvent. The (*S*)-(+)-2-(1-naphthoxy)propanol ((*S*)-**2**) and/or (*R*)-(+)-2-(2-naphthoxy)propanol ((*R*)-**5**) thus obtained each gave a single spot on pre-coated Silica Gel 60 F254 TLC plates (Merck Co., Ltd.).

The culture filtrate after ethereal extraction at pH 10 was acidified to pH 2, and shaken with ether (2 l). The ethereal extract was washed with water and dried over anhydrous Na_2SO_4. The crude product obtained after evaporation of the solvent was purified by silica gel column chromatography, using $CHCl_3$ as the eluting solvent, followed by distillation *in vacuo*. The (*R*)-(−)-2-(1-naphthoxy)propionic acid ((*R*)-**1**) from *Glomerella cingulata* (*Malus* and *Prunus* strains), *G. olivarum* and *G. laeticolor*, and (*S*)-(−)-2-(2-naphthoxy)propionic acid ((*S*)-**4**) from *Glomerella cingulata* (*Malus* strain) and *G. olivarum* were thus obtained in a pure state, and each showed a single spot

on pre-coated Silica Gel 60 F254 TLC plates.

b) (*S*)-(+)-2-(1-Naphthoxy)propanol ((*S*)-**2**): Compound (*S*)-**2** showed a single peak on GLC analysis (t_R=2.1 min; column temp. 200 °C isothermal). MS *m/z*: Calcd for $C_{13}H_{14}O_2$ (M^+) 202.0982. Found: 202.1003. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2950, 1575, 1395, 1265, 1200, 1030, 925, 715. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, d, J=6.1 Hz, CH_3), 2.08 (1H, brs, OH), 3.86 (2H, d, J=5.4 Hz, CH_2), 4.71 (1H, m, CH), 6.85—8.29 (7H, m, ArH).

c) (*R*)-(−)-2-(1-Naphthoxy)propionic Acid ((*R*)-**1**): Compound ((*R*)-**1**) showed mp 151—152 °C. MS *m/z*: 216 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3500, 3000, 1730, 1580, 1395, 1260, 1205, 1105, 925. ^{1}H-NMR ($CDCl_3$) δ: 1.78 (3H, d, J= 6.8 Hz, CH_3), 4.93 (1H, q, J=6.8 Hz, CH), 5.08 (1H, brs, OH), 6.68—8.37 (7H, m, ArH). The optical purity of (*R*)-**1** was determined by reducing it to (*R*)-**3** *via* (*R*)-**2** and measuring the integrated areas of the (*R*)-OMe *vs.* (*S*)-OMe signals in the ^{1}H-NMR spectra of (*R*)-**3** in the presence of Eu(fod)$_3$. The results are listed in Table I.

d) (*R*)-(+)-2-(2-Naphthoxy)propanol ((*R*)-**5**): Compound (*R*)-**5** showed a single peak on GLC analysis (t_R=2.6 min; column temp. 200 °C isothermal). MS *m/z*: Calcd for $C_{13}H_{14}O_2$ (M^+) 202.0987. Found: 202.1004. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3125, 3075, 2925, 1595, 1460, 1200, 1040. ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, d, J=6.1 Hz, CH_3), 2.12 (1H, brs, OH), 3.78 (2H, d, J=5.1 Hz, CH_2), 4.65 (1H, m, CH), 7.08—7.79 (7H, m, ArH).

e) (*S*)-(−)-2-(2-Naphthoxy)propionic Acid ((*S*)-**4**): Compound ((*S*)-**4**) showed mp 107—108 °C. MS *m/z*: 216 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3030, 2935, 1735, 1638, 1610, 1520, 1475, 1210, 1040, 725. ^{1}H-NMR ($CDCl_3$) δ: 1.75 (3H, d, J= 6.8 Hz, CH_3), 4.86 (1H, q, J=6.8 Hz, CH), 5.20 (1H, brs, OH), 7.08—7.79 (7H, m, ArH). The optical purity of (*S*)-**4** was determined by reducing it to (*S*)-**6** *via* (*S*)-**5** and measuring the integrated areas of the (*R*)-OMe *vs.* (*S*)-OMe signals in the ^{1}H-NMR spectra of (*S*)-**6** in the presence of Eu(fod)$_3$. The results are listed in Table I.

Chemical Reduction of (*R*)-(−)-2-(1-Naphthoxy)propionic Acid ((*R*)-1) to (*R*)-2-(1-Naphthoxy)propanol ((*R*)-2) ——A solution of (*R*)-**1** (100 mg) in dry ether (4 ml) was added to a suspension of $LiAlH_4$ (40 mg) in dry ether (4 ml), and the mixture was stirred for 2 h at reflux temperature, then cooled. Water was added and the whole was shaken with ether. The ethereal layer was washed with a small amount of saturated aq. NaCl solution, and dried over anhydrous Na_2SO_4, then the solvent was removed. Compound ((*R*)-**2**) was thus obtained in almost quantitative yield, and showed the same IR and ^{1}H-NMR spectra as the corresponding compound ((*S*)-**2**). This compound ((*R*)-**2**) was derived into its (*R*)-(+)-MTPA ester ((*R*)-**3**) as in the case of (±)-**3** from (±)-**2**, in order to determine the optical purity of (*R*)-**2** (hence (*R*)-**1**). The optical purities thus determined of (*R*)-**1** obtained with *G. olivarum*, *G. laeticolor*, *Glomerella cingulata* (*Malus* and *Prunus* strains) are shown in Table I.

Chemical Reduction of (*S*)-(−)-2-(2-Naphthoxy)propionic Acid ((*S*)-4) to (*S*)-2-(2-Naphthoxy)propanol ((*S*)-5) ——The acid ((*S*)-**4**) was derived into the alcohol ((*S*)-**5**) by the use of LAH as in the case of (*R*)-**2** from (*R*)-**1**. Yields were almost quantitative. IR and ^{1}H-NMR spectra were the same as those of the corresponding compound ((*R*)-**5**). This compound ((*S*)-**5**) was derived into its (*R*)-(+)-MTPA ester ((*S*)-**6**) as in the case of the (±)-**3** from (±)-**2**, in order to determine the optical purity of (*S*)-**5** (hence (*S*)-**4**). The optical purities thus determined of (*S*)-**4** obtained with *G. olivarum* and *Glomerella cingulata* (*Malus* strain) are shown in Table I.

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Simple Preparation of 3-Oxoalkyltriphenylphosphonium Salts Effected by Using 2,6-Lutidinium Salts

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Michael addition of triphenylphosphine to α,β-unsaturated carbonyl compounds was effected in dichloromethane by the action of 2,6-lutidinium salts to give the corresponding 3-oxoalkyltriphenylphosphonium salts.

Keywords——3-oxoalkylphosphonium salt; triphenylphosphine; α,β-unsaturated carbonyl compound; Michael addition; 2,6-lutidinium salt

In the course of studies on the addition reactions of electrochemically generated triphenylphosphine radical cation ($Ph_3P^{+\cdot}$),[1] we have shown that 1-cycloalkenyltriphenylphosphonium salts can be prepared easily by electrochemical oxidation of triphenylphosphine (**1**) in the presence of cycloalkenes by using 2,6-lutidinium perchlorate as the supporting electrolyte.[2] Electrolysis of **1** in the presence of allylsilanes, electron-rich olefins, has been found to give allylphosphonium salts.[3] On the other hand, we show here that the addition of **1** to α,β-unsaturated carbonyl compounds (**2**), electron-deficient olefins, can be effected without electrolysis, that is, 3-oxoalkyltriphenylphosphonium salts (**3**) were obtained simply by mixing **1** and **2** at ambient temperature when the lutidinium salt was present in the system (Chart 1). The phosphonium salts **3** can be regarded as synthetic equivalents of β-acylethyl and β-acylvinyl anions.[4]

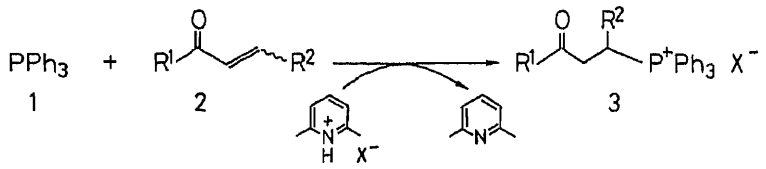

Chart 1

Although Michael addition of **1** to **2** in the presence of a strong acid such as hydrobromic acid has been reported,[5] formation of 3 under neutral conditions is not known. Thus, the reaction in Chart 1 caused by the lutidinium salt (a very weak acid) should be an efficient method for the preparation of **3** under mild conditions. A similar addition reaction of **1** to α,β-unsaturated ketones aided by *tert*-butyldimethylsilyl triflate has been reported recently to give the corresponding *O*-silylated enol phosphonium salts.[6]

When an equimolar mixture of **1**, **2**, and 2,6-lutidinium perchlorate or tetrafluoroborate in dichloromethane was stirred for 10 h, the corresponding phosphonium salts **3** were produced: both of the lutidinium salts were equally effective for the reaction. The results are summarized in Table I. The yields of **3** by the present method are comparable to those observed under strongly acidic conditions.[5] In addition, the products were often isolated as

TABLE I. Preparation of 3, $[R^1COCH_2CH(R^2)\text{-}P^+Ph_3X^-]$[a]

	R^1	R^2	X	Yield (%)[b]		R^1	R^2	X	Yield (%)[b]
3a	EtO	H	ClO_4	83	**3e**	$-(CH_2)_3-$		ClO_4	67
3a'	EtO	H	BF_4	82	**3f**	$-(CH_2)_2-$		ClO_4	63
3b	H	H	ClO_4	79	**3g**	$-O-(CH_2)_2-$		BF_4	61
3c	Me	H	ClO_4	72	**3h**	NH_2	H	ClO_4	55
3d	Et	H	BF_4	84	**3i**	CN for R^1CO	H	ClO_4	61

a) General procedure, see Experimental. *b*) Isolated yield.

hydrates in the latter case, but not in the present case.

Preparation of **3** was also conducted in acetonitrile, but the yield was lower than that in dichloromethane: for example, the yield of **3a** was 76% in acetonitrile (*cf.* Table I). Preliminary experiments were carried out to see whether other salts of weak bases can be used for the reaction. Acetonitrile was employed as the medium because the salts were insoluble in dichloromethane. Pyridinium tetrafluoroborate was as effective as the lutidinium salts: the yield of **3a'** was 76%. Methylammonium perchlorate, selected as one of the typical perchlorate salts derived from aliphatic amines, was less effective: the yield of **3a** was 55%. Although a weak organic acid such as acetic acid did not promote the reaction, addition of sodium perchlorate to the system resulted in the formation of **3** in 30—70% yields. Thus, in acetonitrile, various salts can be used as reagents for the reaction of **1** and **2**. However, the dichloromethane–2,6-lutidinium salt system seems to give better yields of **3**.

Experimental

All melting points are uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on JASCO A-202 and Hitachi R-20 spectrometers, respectively.

Materials——2,6-Lutidinium perchlorate and tetrafluoroborate were prepared by adding a 70% solution of $HClO_4$ (160 g) and a 42% solution of HBF_4 (210 g), respectively, to 2,6-lutidine (110 g) at 0 °C; both salts were recrystallized from AcOEt–EtOH, and stored over P_2O_5. Pyridinium tetrafluoroborate and methylammonium perchlorate were prepared similarly and recrystallized from EtOH and EtOH–ether, respectively.

General Procedure for the Preparation of 3-Oxoalkylphosphonium Salts (3)——A solution of **1** (3 mmol), **2** (3 mmol), and 2,6-lutidinium perchlorate or tetrafluoroborate (3 mmol) in CH_2Cl_2 (10 ml) was stirred at room temperature for 10 h. The reaction mixture was poured into water (100 ml) and extracted with $CHCl_3$ (3 × 30 ml). The organic layer was dried over anhydrous $MgSO_4$ and concentrated to *ca.* 1 ml under reduced pressure. The phosphonium salt **3** separated out when the residue was added dropwise to ether (50 ml) with stirring. The following compounds were obtained in this manner.

2-Carbethoxyethyltriphenylphosphonium Perchlorate (**3a**): mp 146—147 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730, 1100. NMR ($CDCl_3$) δ: 1.15 (3H, t, J=7 Hz), 2.5—3.1 (2H, m), 3.4—3.7 (2H, m), 4.95 (2H, q, J=7 Hz), 7.5—8.0 (15H, m). *Anal.* Calcd for $C_{23}H_{24}ClO_6P$: C, 59.68; H, 5.23; Cl, 7.66. Found: C, 59.86; H, 5.16; Cl, 7.70.

2-Carbethoxyethyltriphenylphosphonium Tetrafluoroborate (**3a'**): mp 118—119 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730. NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7 Hz), 2.5—3.1 (2H, m), 3.3—4.1 (4H, m), 7.2—8.2 (15H, m). *Anal.* Calcd for $C_{23}H_{24}BF_4O_2P$: C, 61.36; H, 5.37. Found: C, 61.21; H, 5.36.

2-Formylethyltriphenylphosphonium Perchlorate (**3b**): mp 132—134 °C (from $CHCl_3$–AcOEt). IR ν_{max}^{Nujol} cm^{-1}: 1730, 1100. NMR (CD_3CN) δ: 2.5—4.0 (4H, m), 7.5—8.1 (15H, m), 9.60 (1H, brd, J=4 Hz). *Anal.* Calcd for $C_{21}H_{20}ClO_5P$: C, 60.23; H, 4.81; Cl, 8.47. Found: C, 59.96; H, 4.82; Cl, 8.79.

2-Acetylethyltriphenylphosphonium Perchlorate (**3c**): mp 166—168 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1100. NMR ($CDCl_3$) δ: 2.10 (3H, s), 2.9—3.8 (4H, m), 7.4—7.9 (15H, m). *Anal.* Calcd for $C_{22}H_{22}ClO_5P$: C, 61.05; H, 5.12; Cl, 8.19. Found: C, 60.88; H, 5.06; Cl, 8.06.

2-Propionylethyltriphenylphosphonium Tetrafluoroborate (**3d**): mp 202—203 °C (from $CHCl_3$–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720. NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7 Hz), 2.38 (2H, q, J=7 Hz), 2.7—3.2 (2H, m), 3.3—3.8 (2H, m), 7.4—8.0 (15H, m). *Anal.* Calcd for $C_{23}H_{24}BF_4O$: C, 63.62; H, 5.57. Found: C, 63.68; H, 5.48.

3-Oxocyclohexyltriphenylphosphonium Perchlorate (**3e**): mp 212—215 °C (from CH_2Cl_2–AcOEt). IR ν_{max}^{Nujol} cm^{-1}: 1720, 1100. NMR (DMSO-d_6) δ: 1.7—2.7 (8H, m), 4.7 (1H, m), 7.6—8.2 (15H, m). *Anal.* Calcd for $C_{24}H_{24}ClO_5P$: C, 62.82; H, 5.27; Cl, 7.73. Found: C, 62.84; H, 5.22; Cl, 7.81.

3-Oxocyclopentyltriphenylphosphonium Perchlorate (**3f**): mp 155—158 °C (from CH_2Cl_2–AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1755, 1100. NMR ($CDCl_3$) δ: 1.5—3.0 (6H, m), 4.90 (1H, m), 7.5—8.2 (15H, m). *Anal.* Calcd for $C_{23}H_{22}ClO_5P$: C, 62.16; H, 4.98; Cl, 7.98. Found: C, 62.06; H, 4.85; Cl, 8.00.

4-(Tetrahydro-2-pyronyl)triphenylphosphonium Tetrafluoroborate (**3g**): mp 209—211 °C (from CH_3CN–ether). IR ν_{max}^{Nujol} cm^{-1}: 1740. NMR (CD_3CN) δ: 2.2—3.4 (4H, m), 4.0—4.8 (3H, m), 7.6—8.1 (15H, m). *Anal.* Calcd for $C_{23}H_{22}BF_4O_2$: C, 61.63; H, 4.95. Found: C, 61.78; H, 4.93.

2-Carbamylethyltriphenylphosphonium Perchlorate (**3h**): mp 183—185 °C (from CH_3CN–ether). IR ν_{max}^{Nujol} cm^{-1}: 1675, 1630, 1100. NMR (CD_3CN) δ: 2.2—2.8 (2H, m), 3.3—3.8 (2H, m), 5.5—6.5 (2H, br, NH_2), 7.5—8.0 (15H, m). *Anal.* Calcd for $C_{21}H_{21}ClNO_5P$: C, 58.14; H, 4.88; Cl, 8.17; N, 3.23. Found: C, 58.31; H, 4.81; Cl, 8.00; N, 3.14.

2-Cyanoethyltriphenylphosphonium Perchlorate (**3i**): mp 147—149 °C (from CH_3CN–ether). IR ν_{max}^{Nujol} cm^{-1}: 2250, 1100. NMR (DMSO-d_6) δ: 2.7—3.3 (4H, m), 3.8—4.4 (3H, m), 7.5—8.1 (15H, m). *Anal.* Calcd for $C_{21}H_{19}ClNO_4P$: C, 60.66; H, 4.61; Cl, 8.53; N, 3.37. Found: C, 60.58; H, 4.53; Cl, 8.74; N, 3.29.

The reaction in acetonitrile was carried out similarly. Thus, a solution of **1** (3 mmol), **2** (3 mmol), and a particular salt (3 mmol) or AcOH (3 mmol) and $NaClO_4$ (3 mmol) in CH_3CN (10 ml) was stirred at room temperature for 10 h. The phosphonium salts **3** were isolated by essentially the same procedure as described above.

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Studies on Peptides. CL.[1,2] Syntheses of [D-His^2]-Analogs of Enkephalin and Adrenorphin and Several [D-Arg^2]enkephalin Analogs

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[D-His^2]Leu-enkephalin and [D-His^2]adrenorphin were synthesized. In addition, eight [D-Arg^2]enkephalin-related peptides were synthesized. Their inhibitory effects on electrically stimulated myenteric plexus-longitudinal muscle preparations of guinea-pig ileum were examined.

Keywords——[D-His^2]enkephalin; [D-His^2]adrenorphin; [D-Arg^2]enkephalin analog; μ receptor; guinea-pig ileum assay; trifluoromethanesulfonic acid deprotection; methanesulfonic acid deprotection

Replacement of the Gly^2 residue of enkephalin[3] and related peptides by D-amino acids, such as D-Ala,[4] or D-Met,[5] or D-Met(O)[6] or D-Arg,[7,8] is known to bring about considerably higher analgesic activity. We therefore examined the biological activities of eight [D-Arg^2]enkephalin-related peptides, together with those of [D-His^2]-analogs of enkephalin and adrenorphin,[9] possessing different basicity from that of [D-Arg^2]-derivatives.

A [D-His^2]-analog of Leu-enkephalin (**1**) was synthesized by successive azide (Az) condensations[10] of Z(OMe)–D-His–$NHNH_2$ and Z(OMe)–Tyr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Gly–Phe–Leu–OBzl,[11] followed by removal of the two protecting groups from the resulting protected pentapeptide, *i.e.*, the Bzl group by catalytic hydrogenolysis, then the Z(OMe) group readily by TFA treatment. The deprotected peptide was purified by partition chromatography[12] on Sephadex G-10, followed by high-performance liquid chromatography (HPLC).

[D-His^2]adrenorphin (**2**) was similarly prepared by succesive Az condensations of Z(OMe)–D-His–$NHNH_2$ and Z(OMe)–Tyr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Gly–Phe–Met(O)–Arg(Mts)–Arg(Mts)–Val–NH_2, followed by deprotection of the resulting protected octapeptide with 1 M TFMSA–thioanisole/TFA.[13] The above amino component was obtained by the Su condensation[14] of Z(OMe)–Gly–OH with a TFA-treated sample of Z(OMe)–Phe–Met(O)–Arg(Mts)–Arg(Mts)–Val–NH_2, an intermediate of our previous synthesis of adrenorphin.[11] After incubation with dithiothreitol, the desired peptide was purified by partition chromatography on Sephadex G-10, followed by HPLC.

Next, we prepared five [D-Arg^2]enkephalin-related peptides, **3** to **7**. The C-terminal end of [D-Arg^2]Leu-enkephalin (in one instance, Met-enkephalin) was extended by Arg, Arg–Arg, Arg–Arg–Ile, and Arg–Arg–Ile–Arg, respectively, to examine the effects due to such basic extensions. These compounds can be classified as analogs of kyotorphin[15] or adrenorphin or the N-terminal portion of dynorphin.[16]

H–Tyr–D-Arg–Gly–Phe–Leu–Arg–OH (**3**) was prepared starting from H–Arg(NO_2)–OBzl. Z(OMe)–Leu–Arg(NO_2)–OBzl was prepared by the PCP procedure[17] and this, after TFA treatment, was condensed with a newly prepared dipeptide hydrazide, Boc–Gly–Phe–$NHNH_2$, *via* the Az to give Boc–Gly–Phe–Leu–Arg(NO_2)–OBzl. This tetrapeptide chain was elongated by two successive condensations of Z(OMe)–D-Arg(NO_2)–OH *via* the mixed anhydride (MA)[18] and Z(OMe)–Tyr(Bzl)–OH *via* the TCP ester[19] to give Z(OMe)–Tyr(Bzl)–D-Arg(NO_2)–Gly–Phe–Leu–Arg(NO_2)–OBzl, from which all protecting groups were removed by catalytic hydrogenolysis. The product (**3**) was purified by ion-exchange chromatography on CM cellulose, followed by gel-filtration on Sephadex G-10, as was done with other analogs.

Next, considering convenient procedures for the syntheses of analogous compounds, we decided to construct the common sequence, Tyr–D-Arg–Gly–Phe, by the Az condensations of two units, Boc–Gly–Phe–$NHNH_2$ and Z–Tyr–D-Arg(Mts)–$NHNH_2$. Only C-terminal portions were prepared for each analog. Thus, H–Tyr–D-Arg–Gly–Phe–Met–Arg–OH· (**4**) was prepared starting from Z(OMe)–Met–Arg(Mts)–OH obtained by the TCP procedure. Z–Tyr–D-Arg(Mts)–Gly–Phe–Met–Arg(Mts)–OH thus obtained was treated with MSA[20] to afford **4**.

For the preparation of H–Tyr–D-Arg–Gly–Phe–Leu–Arg–Arg–OH (**5**), Z(OMe)–Leu–Arg(Mts)–$NHNH_2$, obtained by PCP condensation of Z(OMe)–Leu–OH and H-Arg(Mts)–OMe, followed by the usual hydrazine treatment, was condensed with H–Arg(Mts)–OH to give Z(OMe)–Leu–Arg(Mts)–Arg(Mts)–OH. Chain elongation of this tripeptide was carried out as described above, then all protecting groups employed were cleaved by 1 M TFMSA–thioanisole in TFA to give **5**. Next, Z(OMe)–Leu–Arg(Mts)–$NHNH_2$ obtained above was used to prepare the C-terminal portion of H–Tyr–D-Arg–Gly–Phe–Leu–Arg–Arg–Ile–OH (**6**). The necessary C-terminal tetrapeptide unit was obtained by Az condensation of the above hydrazide with a TFA-treated sample of Z(OMe)–Arg(Mts)–Ile–OBzl. Subsequent chain elongation and deprotection were carried out as described above to give **6**. Next, Z(OMe)–Arg(Mts)–Ile–OBzl obtained above was used to prepare H–Tyr–D-Arg–Gly–Phe–Leu–Arg–Arg–Ile–Arg–OH (**7**). This dipeptide ester was converted to the corresponding hydrazide, then condensed with H–Arg(Mts)–OH to give Z(OMe)–Arg(Mts)–Ile–Arg(Mts)–OH. The rest of the reactions were performed as described above to give **7**.

Next, three analogs of [D-Arg^2]Leu-enkephalin, H–Tyr–D-Arg–Gly–Phe–Leu–R [R = D-Arg–OH (**8**), Arg-ol (**9**), and Arg–NH_2 (**10**)], were prepared. For these syntheses, an available tripeptide, Boc–Gly–Phe–Leu–OH,[8] was converted to the corresponding hydrazide *via* the methyl ester. Z(OMe)–Gly–Phe–Leu–$NHNH_2$ thus obtained was used to prepare the respective C-terminal portions of these analogs. For the preparation of compound (**8**), this tripeptide hydrazide was condensed with H–D-Arg(Mts)–OH and the resulting protected tetrapeptide, after TFA treatment, was condensed with Z–Tyr–D-Arg(Mts)–$NHNH_2$. All protecting groups were cleaved from the resulting hexapeptide by 1 M TFMSA–thioanisole in TFA and the deprotected peptide was purified by ion-exchange chromatography on CM-cellulose, followed by gel-filtration on Sephadex G-10 as stated above. Using H–Arg(Mts)–NH_2, compound (**10**) was similarly prepared. For the preparation of compound (**9**), Boc–Gly–Phe–Leu–Arg(Mts)–ol was obtained by reduction of Boc–Gly–Phe–Leu–Arg(Mts)–OMe with $NaBH_4$.[21] Subsequent chain elongation and deprotection were carried out as described above.

In the present investigations, electrically stimulated myenteric plexus-longitudinal muscle preparations of guinea-pig ileum,[22] a typical μ receptor preparation,[23] were used to test the biological activities of synthetic peptides. The volume of bath fluid was 1.5 ml. In each preparation, the inhibitory effects of Met-enkephalin and a synthetic peptide were examined in turn, and the relative potency was calculated as shown in Table I. In this assay system, only

TABLE I. Relative Potencies of Inhibitory Effects of Synthetic Peptides on Guinea-Pig Ileum

		Relative potencies
1	H-Tyr-D-His-Gly-Phe-Leu-OH	0.17
2	H-Tyr-D-His-Gly-Phe-Met-Arg-Arg-Val-NH_2	2.04
3	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-OH	3.30
4	H-Tyr-D-Arg-Gly-Phe-Met-Arg-OH	0.50
5	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-Arg-OH	0.38
6	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-Arg-Ile-OH	1.00
7	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH	0.24
8	H-Tyr-D-Arg-Gly-Phe-Leu-D-Arg-OH	0.36
9	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-ol	0.07
10	H-Tyr-D-Arg-Gly-Phe-Leu-Arg-NH_2	0.14
	H-Tyr-Gly-Gly-Phe-Leu-OH (Leu-enkephalin)	0.50
	H-Tyr-Gly-Gly-Phe-Met-OH (Met-enkephalin)	1.00

[D-His^2]adrenorphin and the analog **3** exhibited higher activities than Leu-enkephalin, but no enhancement of biological activity was observed in the other compounds. Further accumulation of experimental results seems necessary for a better understanding of the subtle relationship between opioid receptors and enkephalin.

Experimental

General experimental methods employed in this investigation are essentially the same as described in our previous synthesis of [D-Arg^2]enkephalin.[7] *Rf* values in thin layer chromatography (TLC), performed on silica gel (Kieselgel G, Merck), refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1), and Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2). HPLC was conducted with a Waters 204 compact model equipped with a Nucleosil 5C18 column (4×150 mm).

Synthesis of [D-His^2]Leu-Enkephalin

Z(OMe)–D-His–Gly–Phe–Leu–OBzl——The Az [prepared from 2.36 g (7.0 mmol) of Z(OMe)-D-His $NHNH_2$] in DMF (25 ml) and Et_3N (1.82 ml, 13.0 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)-Gly-Phe-Leu-OBzl (3.50 g, 6.0 mmol) in DMF (30 ml), then the mixture was stirred at 4 °C overnight and the solvent was removed by evaporation. The residue was treated with AcOEt and the resulting powder was recrystallized from MeOH and ether; yield 2.30 g (54%), mp 100—105 °C, $[\alpha]_D^{15}$ −19.6° (*c*=1.0, MeOH), Rf_1 0.74. *Anal.* Calcd for $C_{39}H_{46}N_6O_8 \cdot 2H_2O$: C, 61.40; H, 6.61; N, 11.02. Found: C, 61.63; H, 6.33; N, 11.44.

Z(OMe)–Tyr–D-His–Gly–Phe–Leu–OBzl——The Az [prepared from 0.82 g (2.3 mmol) of Z(OMe)-Tyr-$NHNH_2$] in DMF (5 ml) and Et_3N (0.59 ml, 4.2 mmol) were added to a TFA-treated sample of the above protected tetrapeptide (1.36 g, 1.9 mmol) in DMF (10 ml), then the mixture was stirred at 4 °C overnight and the solvent was removed by evaporation. The residue was treated with AcOEt and the resulting powder was recrystallized from MeOH and ether; yield 0.91 g (55%), mp 114—118 °C, $[\alpha]_D^{15}$ −16.1° (*c*=1.0, MeOH), Rf_1 0.76. *Anal.* Calcd for $C_{48}H_{55}N_7O_{10} \cdot H_2O$: C, 63.49; H, 6.33; N, 10.80. Found: C, 63.55; H, 6.30; N, 11.16.

H–Tyr–D-His–Gly–Phe–Leu–OH (1)——The above protected pentapeptide (250 mg, 0.28 mmol) in MeOH (20 ml) was hydrogenated over a Pd catalyst for 5 h, then the catalyst was removed by filtration. The filtrate was concentrated and dry ether was added. The resulting powder was next treated with TFA (1.0 ml) in the presence of thioanisole (0.11 ml) in an ice-bath for 60 min. Dry ether was added and the resulting power was purified by partition chromatography on Sephadex G-10 (3.0×100 cm), equilibrated with the lower phase of *n*-BuOH–AcOH–H_2O (4:1:5). The column was eluted with the upper phase of the above solvent. The desired fractions (10 ml each, tube Nos. 40—60, monitored by measuring the ultraviolet (UV) absorption at 275 nm) were combined and the solvent was removed by evaporation. The residue was lyophilized to give a fluffy powder; yield 102 mg (57%). The sample (purity 97%, determined by analytical HPLC) was submitted to bioassay. For characterization, a part of the product (25 mg) was purified by repeated HPLC using isocratic elution with 15% MeCN in 0.1% TFA. The solvent of the desired eluate (retention time, 7.5 min) was evaporated off, and the residue was lyophilized. Yield 18 mg (71%), $[\alpha]_D^{15}$ −2.9° (*c*=1.1, MeOH), Rf_1 0.21. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table II, together with those of other peptides. *Anal.* Calcd for $C_{32}H_{41}N_7O_7 \cdot 2CF_3COOH \cdot 4H_2O$: C, 46.20; H, 5.49; N, 10.48. Found: C, 46.09; H,

TABLE II. Amino Acid Ratios in 6 N HCl Hydrolysates of Enkephalin Analogs

	Analogs	Tyr Y	Gly G	Phe F	Leu L	Met M	Val V	Ile I	His H	Arg R	Rec. %
1	H-(Y-H-G-F-L)-OH	0.88	1.00	0.94	0.93				0.88		87
2	H-(Y-H-G-F-M-R-R-V)-NH_2	0.89	1.00	0.98		0.82	0.97		0.94	1.93	72
3	H-(Y-R-G-F-L-R)-OH	1.02	1.00	0.95	1.05					1.97	87
4	H-(Y-R-G-F-M-R)-OH	0.97	1.00	1.04		0.95				1.94	84
5	H-(Y-R-G-F-L-R-R)-OH	0.94	1.00	1.03	1.04					2.88	85
6	H-(Y-R-G-F-L-R-R-I)-OH	1.03	1.00	1.00	0.96			0.97		2.96	80
7	H-(Y-R-G-F-L-R-R-I-R)-OH	0.89	1.00	0.96	1.03			0.93		4.03	82
8	H-(Y-R-G-F-L-R)-OH	0.92	1.00	0.98	0.96					2.07	84
9	H-(Y-R-G-F-L-R)-ol	0.93	1.00	0.97	0.93					1.08	79
10	H-(Y-R-G-F-L-R)-NH_2	0.93	1.00	1.07	0.95					1.88	82

Capital letters in parentheses indicate amino acids. Underlining indicates D-amino acids.

TABLE III. Characterization of Enkephalin Analogs, 3 and 4

Compound	Proc.	Yield (%)	mp (°C)	$[\alpha]_D^{24}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(L-R(n))-OBzl	PCP	90	65—67	−14.3°	$C_{28}H_{38}N_6O_8$	57.32	6.53	14.33
						(57.21	6.46	14.01)
Boc-(G-F)-OBzl	DCC	69	80—82	−5.2°	$C_{23}H_{28}N_2O_5$	66.97	6.84	6.79
						(67.36	6.78	6.91)
Boc-(G-F)-$NHNH_2$	NH_2NH_2	85	180—185	−3.3°	$C_{16}H_{24}N_4O_4$	57.13	7.19	16.66
						(57.48	7.36	16.51)
Boc-(G-F-L-R(n))-OBzl	Az	87	105—110	−12.5°	$C_{35}H_{50}N_8O_9$	57.83	6.93	15.42
						(57.63	6.85	15.00)
Z(OMe)-(R(n)-G-F-L-R(n))-OBzl	MA	90	104—107	−8.2°	$C_{45}H_{61}N_{13}O_{13}\cdot 3H_2O$	51.66	6.45	17.41
						(51.66	5.89	17.22)
Z(OMe)-(Y(b)-R(n)-G-F-L-R(n))-OBzl	TCP	97	105—110	−14.0°	$C_{61}H_{76}N_{14}O_{15}\cdot H_2O$	57.99	6.22	15.52
						(57.96	6.04	15.41)
H-(Y-R-G-F-L-R)-OH (3)	H_2-Pd	43		+18.8° (0.2 N AcOH)	$C_{38}H_{58}N_{12}O_8\cdot 3AcOH\cdot 3H_2O$	50.56	7.33	16.08
						(50.79	6.98	16.23)
Z(OMe)-(M-R(m))-OH	TCP	75	88—92	−9.1°	$C_{29}H_{41}N_5O_8S_2$	53.44	6.14	10.75
						(53.51	6.22	10.74)
Boc-(G-F-M-R(m))-OH	Az	87	127—132	−2.9°	$C_{36}H_{53}N_7O_9S_2\cdot 0.5H_2O$	53.97	6.80	12.24
						(53.77	6.61	12.16)
Z-(Y-R(m)-G-F-M-R(m))-OH	Az	63	139—144	−11.6°	$C_{63}H_{82}N_{12}O_{14}S_3$	56.99	6.23	12.66
						(56.56	6.22	12.35)
H-(Y-R-G-F-M-R)-OH (4)	MSA	34		+18.0° (0.2 N AcOH)	$C_{37}H_{56}N_{12}O_8S\cdot 3AcOH\cdot 3H_2O$	48.57	7.02	15.81
						(48.05	6.61	15.74)

TABLE IV. Characterization of Enkephalin Analogs, 5 and 6

Compound	Proc.	Yield (%)	mp (°C)	$[\alpha]_D^{24}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(L-R)-OMe	PCP	87	65—70	−11.2°	$C_{31}H_{45}N_5O_6S \cdot H_2O$	55.92 (55.97	7.12 6.72	10.52 10.20)
Z(OMe)-(L-R)-$NHNH_2$	NH_2NH_2	91	95—97	−13.5°	$C_{30}H_{45}N_7O_7S \cdot 0.5H_2O$	54.86 (54.88	7.06 6.64	14.93 14.88)
Z(OMe)-(L-R-R)-OH	Az	95	115—118	−2.4°	$C_{45}H_{65}N_9O_{11}S_2$	55.59 (56.16	6.74 6.76	12.97 12.93)
Z(OMe)-(G-F-R-R)-OH	Az	92	142—146	−7.6°	$C_{52}H_{77}N_{11}O_{12}S_2 \cdot H_2O$	55.25 (55.20	7.04 6.97	13.63 13.27)
Z-(Y-R-G-F-L-R-R)-OH	Az	88	129—133	+1.0°	$C_{79}H_{106}N_{16}O_{17}S_3 \cdot 1.5H_2O$	56.64 (56.38	6.56 6.71	13.38 13.15)
H-(Y-R-G-F-L-R-R)-OH (5)	TFMSA	54		+8.3° 0.2 N AcOH	$C_{44}H_{70}N_{16}O_9 \cdot 4AcOH \cdot 2H_2O$	50.23 (50.25	7.30 7.29	18.03 18.22)
Z(OMe)-(R-I)-OBzl	DCC	83	45—50	−7.5°	$C_{37}H_{49}N_5O_8S$	61.39 (61.80	6.82 6.82	9.68 9.42)
Z(OMe)-(L-R-R-I)-OBzl	Az	72	110—115	−20.9°	$C_{58}H_{82}N_{10}O_{12}S \cdot H_2O$	58.36 (58.41	7.09 6.96	11.74 11.74)
Z(OMe)-(G-F-L-R-R-I)-OBzl	Az	84	126—131	−12.4°	$C_{65}H_{94}N_{12}O_{13}S_2 \cdot H_2O$	58.53 (58.52	7.26 7.01	12.60 12.69)
Z-(Y-R-G-F-L-R-R-I)-OBzl	Az	97	203—207	−8.4°	$C_{92}H_{123}N_{17}O_{18}S_3$	58.83 (58.56	6.76 6.68	12.68 12.86)
H-(Y-R-G-F-L-R-R-I)-OH (6)	TFMSA	46		−3.0° 0.2 NAcOH	$C_{50}H_{81}N_{17}O_{10} \cdot 4AcOH \cdot 2.5H_2O$	51.01 (50.77	7.53 7.38	17.44 17.20)

5.53; N, 10.52.

Synthesis of [D-His^2]adrenorphin

Z(OMe)-Gly-Phe-Met(O)-Arg(Mts)-Arg(Mts)-Val-NH_2——A mixture of Z(OMe)-Gly-OSu (3.10 g, 12.9 mmol), Et_3N (3.46 ml, 24.7 mmol) and a TFA-treated sample of Z(OMe)-Phe-Met(O)-Arg(Mts)-Arg(Mts)-Val-NH_2 (14.73 g, 11.8 mmol) in DMF (80 ml) was stirred at room temperature overnight. The solvent was removed by evaporation and the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and recrystallized from MeOH and ether; yield 8.36 g (54%), mp 170—171 °C, $[\alpha]_D^{19}$ −8.8° (c=0.9, DMF), Rf_1 0.64. *Anal.* Calcd for $C_{60}H_{85}N_{13}O_{14}S_3$: C, 55.07; H, 6.55; N, 13.92. Found: C, 54.78; H, 6.44; N, 13.97.

Z(OMe)-D-His-Gly-Phe-Met(O)-Arg(Mts)-Arg(Mts)-Val-NH_2——The Az [prepared from 1.26 g (3.8 mmol) of Z(OMe)-D-His-$NHNH_2$] in DMF (10 ml) and Et_3N (0.58 ml, 4.1 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above hexapeptide amide (3.79 g, 2.9 mmol) in DMF (15 ml) containing Et_3N (0.4 ml, 2.9 mmol), and the solution was stirred at 4 °C overnight. After evaporation of the solvent, the residue was treated as stated above and precipitated from DMF with MeOH; yield 2.86 g (68%), mp 179—181 °C, $[\alpha]_D^{19}$ −7.9° (c=0.5, DMF), Rf_1 0.38. *Anal.* Calcd for $C_{66}H_{92}N_{16}O_{15}S_3 \cdot 2H_2O$: C, 53.49; H, 6.53; N, 15.10. Found: C, 53.59; H, 6.43; N, 14.78.

Z(OMe)-Tyr-D-His-Gly-Phe-Met(O)-Arg(Mts)-Arg(Mts)-Val-NH_2——The Az [prepared from 0.83 g (2.3 mmol) of Z(OMe)-Tyr-$NHNH_2$] in DMF (10 ml) and Et_3N (0.35 ml, 2.5 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above heptapeptide amide (2.56 g, 1.8 mmol) in DMF (10 ml) containing

TABLE V. Characterization of Enkephalin Analogs, 7 and 8

Compound	Proc.	Yield (%)	mp (°C)	$[\alpha]_D^{24}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(R(m)-I)-NHNH$_2$	NH_2NH_2	93	135—138	+4.7°	$C_{30}H_{45}N_7O_7S$	55.62 (55.50	7.00 6.97	15.14 14.99)
Z(OMe)-(R(m)-I-R(m))-OH	Az	89	125—129	−13.6°	$C_{45}H_{65}N_9O_{11}S_2 \cdot 0.5H_2O$	55.08 (55.22	6.78 6.84	12.85 12.43)
Z(OMe)-(L-R(m)-R(m)-I-R(m))-OH	Az	91	132—136	−15.6°	$C_{66}H_{98}N_{14}O_{15}S \cdot 1.5H_2O$	54.64 (54.55	7.02 7.02	13.52 13.54)
Z(OMe)-(G-F-L-R(m)-R(m)-I-R(m))-OH	Az	81	155—159	−4.0°	$C_{73}H_{110}N_{16}O_{16}S_3 \cdot 2H_2O$	54.80 (54.71	7.18 6.98	14.01 14.04)
Z-(Y-R(m)-G-F-L-R(m)-R(m)-I-R(m))-OH	Az	84	164—167	+0.9°	$C_{100}H_{139}N_{21}O_{21}S_4 \cdot 1.5H_2O$	56.48 (56.40	6.73 6.78	13.83 13.80)
H-(Y-R-G-F-L-R-R-I-R)-OH (7)	TFMSA	51		−11.3° (0.2 N AcOH)	$C_{56}H_{93}N_{21}O_{11} \cdot 5AcOH \cdot 4H_2O$	49.27 (49.46	7.58 7.19	18.28 17.83)
Z(OMe)-(G-F-L)-NHNH$_2$	NH_2NH_2	83	137—142	−21.1°	$C_{26}H_{35}N_5O_6$	60.80 (60.38	6.87 6.78	13.64 13.46)
Z(OMe)-(G-F-L-R(m))-OH	Az	88	129—134	−9.6°	$C_{41}H_{55}N_7O_{10}S$	58.76 (59.04	6.62 6.68	11.70 11.53)
Z-(Y-R(m)-G-F-L-R(m))-OH	Az	90	143—149	−11.3°	$C_{64}H_{84}N_{12}O_{14}S_2 \cdot 0.5H_2O$	58.29 (58.16	6.50 6.75	12.75 12.48)
H-(Y-R-G-F-L-R)-OH (8)	TFMSA	68		+12.0° (0.2 N AcOH)	$C_{38}H_{58}N_{12}O_8 \cdot 2AcOH \cdot 2.5H_2O$	51.68 (52.05	7.33 7.33	17.22 17.44)

Et_3N (0.25 ml, 1.8 mmol), then the solution was stirred at 4 °C overnight and the solvent was removed by evaporation. The residue was purified as stated above; yield 1.52 g (53%), mp 167—169 °C, $[\alpha]_D^{19}$ −15.6° (c=0.5, DMF), Rf_1 0.45. *Anal.* Calcd for $C_{75}H_{101}N_{17}O_{17}S_3 \cdot 3.5H_2O$: C, 53.87; H, 6.51; N, 14.24. Found: C, 53.93; H, 6.34; N, 13.75.

H-Tyr-D-His-Gly-Phe-Met-Arg-Arg-Val-NH$_2$ (2)——The above protected octapeptide amide (100 mg, 62 μmol) was treated with 1 M TFMSA (1.9 ml) in the presence of *m*-cresol (0.1 ml, 15 eq) in an ice-bath for 2 h, then ether was added and the resulting powder was treated with Amberlite CG-4B (acetate form) for 30 min. The resin was removed by filtration, then the filtrate was incubated with dithiothreitol (0.48 g, 50 eq) at 37 °C overnight and the solvent was removed by lyophilization. The product was next purified by gel-filtration on Sephadex G-15 (3.2 × 104 cm) using 0.5 N AcOH as an eluant. The fractions corresponding to the front main peak (monitored by UV absorption measurement at 275 nm) were collected and the solvent was removed by lyophilization. The product was next purified by partition chromatography on Sephadex G-10 (2.5 × 83 cm) using the solvent system of *n*-BuOH–AcOH–H_2O (4 : 1 : 5) as stated above. The desired fractions (5.4 ml each, tube Nos. 104—110, monitored at UV 275 nm) were combined, then the solvent was removed by evaporation and the residue was lyophilized to give a fluffy powder; yield 32 mg (48%). The sample (purity 96%, determined by analytical HPLC) was submitted to bioassay. For characterization, the product was further purified by HPLC using isocratic elution with 20% MeCN in 0.1% TFA; yield 20 mg (64%), $[\alpha]_D^{20}$ −77.9° (c=0.2, MeOH), Rf_3 0.57. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table II. *Anal.* Calcd for $C_{48}H_{73}N_{17}O_9S \cdot 4CF_3COOH \cdot 2.5H_2O$: C, 42.97; H, 5.28; N, 15.21. Found: C, 43.27; H, 5.58; N, 15.45.

Syntheses of [D-Arg^2]enkephalin Analogs

Eight [D-Arg^2]enkephalin analogs were synthesized as described in the text. Amino acid ratios in 6 N HCl hydrolysates of these compounds are listed in Table II. Physical constants and analytical data of these compounds are listed in Tables III to VI, together with those of their intermediates. Capital letters in parentheses indicate amino acids: Y=Tyr, H=His, R=Arg, G=Gly, F=Phe, L=Leu, I=Ile. Small letters at shoulders indicate protecting

TABLE VI. Characterization of Enkephalin Analogs, **9** and **11**

Compound	Proc.	Yield (%)	mp (°C)	$[\alpha]_D^{24}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Boc-(G-F-L-R(m))-OMe	Az	89	104—107	−22.2°	$C_{38}H_{57}N_7O_9S \cdot H_2O$	56.02 (56.76	7.38 7.00	12.17 12.32)
Boc-(G-F-L-R(m))-ol	$NaBH_4$	89	102—105	−17.3°	$C_{37}H_{57}N_7O_8S \cdot H_2O$	57.11 (57.29	7.64 7.31	12.60 12.46)
Z-(Y(m)-R(m)-G-F-L-R)-ol	Az	93	135—139	−9.5°	$C_{64}H_{86}N_{12}O_{13}S_2 \cdot 2H_2O$	57.72 (57.70	6.81 6.38	12.62 12.25)
H-(Y-R-G-F-L-R)-ol (**9**)	TFMSA	33		+20.0° (0.2 N AcOH)	$C_{38}H_{60}N_{12}O_7 \cdot 3AcOH \cdot 2.5H_2O$	51.70 (51.69	7.59 7.32	16.45 16.36)
Boc-(G-F-L-R(m))-NH_2	NH_3	95	129—134	+2.0°	$C_{37}H_{56}N_8O_8S$	57.49 (57.03	7.30 7.21	14.50 14.31)
Z-(Y(m)-R(m)-G-F-L-R)-NH_2	Az	92	139—142	−2.9°	$C_{64}H_{84}N_{12}O_{14}S_2 \cdot H_2O$	57.94 (57.87	6.53 6.80	12.67 13.03)
H-(Y-R-G-F-L-R)-NH_2 (**10**)	TFMSA	47		+15.8° (0.2 N AcOH)	$C_{32}H_{48}N_{12}O_6 \cdot 3AcOH \cdot H_2O$	50.99 (51.43	6.98 7.07	18.78 18.31)

groups.[2] Arrows indicate positions at which reactions took place.

References and Notes

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2) Unless otherwise stated, amino acids used in this report are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl(or b) = benzyl, Mts(or m) = mesitylenesulfonyl, NO_2(or n) = nitro, Su = *N*-hydroxysuccinimidyl, TCP = 2,4,5-trichlorophenyl, PCP = pentachlorophenyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DMF = dimethylformamide, MSA = methanesulfonic acid, CM = carboxymethyl, DCC = dicyclohexylcarbodiimide.
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Studies on the Bacterial Gall of *Myrica rubra*: Isolation of a New [7,0]-Metacyclophan from the Gall and DL-β-Phenyllactic Acid from the Culture of Gall-Forming Bacteria

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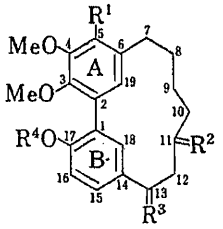
From the bacterial gall of *Myrica rubra*, a new [7,0]-metacyclophan (**1**) was isolated together with three known compounds, myricanone (**2**), porson (**3**) and myricanol (**4**). The structure of the new compound was elucidated to be 5-deoxymyricanone (**1**) on the basis of spectroscopic evidence. The possible intermediacy of **1** in the biosynthesis of **2**, **3** and **4** is discussed. DL-β-Phenyllactic acid was also isolated from the culture of *Pseudomonas syringae* pv. *myricae* pathogenic to the plant.

Keywords——*Myrica rubra*; bacterial gall; 5-deoxymyricanone; myricanol; myricanone; porson; [7,0]-metacyclophan; biogenesis; DL-β-phenyllactic acid; *Pseudomonas syringae* pv. *myricae*

The bacterial gall of *Myrica rubra* SIEB. *et* ZUCC. is formed specifically on the trunk and twigs of the plant by the infection of *Pseudomonas syringae* pv. *myricae* py. nov.[2)] As a part of our chemical studies on the interaction between plants and microorganisms, we have examined the constituents of the bacterial gall of *M. rubra* and have isolated a new [7,0]-metacyclophan (**1**) together with three known compounds, myricanone (**2**),[3,4)] porson (**3**),[5)] and myricanol (**4**).[3,4)] We also examined the metabolites of *P. syringae* pv. *myricae* and isolated DL-β-phenyllactic acid together with L-prolyl-L-tyrosine anhydride (**9**), D-prolyl-L-tyrosine anhydride (**10**), L-prolyl-L-valine anhydride (**11**) and L-prolyl-L-phenylalanine anhydride (**12**), which are presumed to be formed from the nutrient in the medium. This paper describes these results.

The new compound (**1**) from the gall was obtained as an amorphous powder and was optically inactive. The molecular formula was established as $C_{21}H_{24}O_4$ from high-resolution



1: $R^1=R^4=H$, $R^2=O$, $R^3=H_2$
2: $R^1=OH$, $R^2=O$, $R^3=H_2$, $R^4=H$
3: $R^1=OMe$, $R^2=O$, $R^3=H,OH$, $R^4=H$
4: $R^1=OH$, $R^2=H,OH$, $R^3=H_2$, $R^4=H$
5: $R^1=H$, $R^2=O$, $R^3=H_2$, $R^4=COMe$

9: $R^1=\alpha$-H, $R^2=CH_2$-C_6H_4-p-OH
10: $R^1=\beta$-H, $R^2=CH_2$-C_6H_4-p-OH
11: $R^1=\alpha$-H, $R^2=CH(CH_3)_2$
12: $R^1=\alpha$-H, $R^2=CH_2$-C_6H_5

TABLE I. Comparisons of Proton Signals[a] of **1** and Myricanone (**2**) in the Aromatic Region

	1	2
5-H	6.70 (d, J=2 Hz)[b]	
15-H	7.04 (dd, J=8, 3 Hz)	7.06 (dd, J=8, 3 Hz)
16-H	6.84 (d, J=8 Hz)	6.88 (d, J=8 Hz)
18-H	6.75 (d, J=3 Hz)	6.75 (d, J=3 Hz)
19-H	6.52 (d, J=2 Hz)[b]	6.61 (br s)

a) Measured in $CDCl_3$ solution. *b*) The assignment of 19-H in **1** was based on the fact that NOE (12.2%) was observed on irradiation at the frequency at δ 6.75 (18-H).

TABLE II. Results of NOE Experiments for **1** (Measured in C_6D_6 Solution)

Irradiated proton	Protons which showed NOE for irradiated proton (%)	Irradiated proton	Protons which showed NOE for irradiated proton (%)
4-OMe	5-H (18.8)	12-H_2	13-H_2 (6.9)
7-H_2	5-H (5.2)	13-H_2	15-H (7.4)
8-H_2	19-H (6.5)	16-H	15-H (12.6)
9-H_2	19-H (6.5)	18-H	19-H (13.3)
10-H_2	18-H (11.8), 19-H (13.7)	19-H	18-H (13.2)

mass (HR-MS) measurement by electron impact mass spectrometry. Compound **1** gave the monoacetate (**5**) on acetylation and showed absorption maxima at 251 and 295 nm in the ultraviolet (UV) spectrum and absorptions at 3325 (hydroxy), 1705 (ketone), and 1590 and 1505 (benzene ring) cm^{-1} in the infrared (IR) (in $CHCl_3$) spectrum. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) (in $CDCl_3$) spectrum showed signals indicating the presence of two benzene rings, besides the signals due to six methylene, two methoxy carbons (δ 56.1 and 61.4) and a ketonic carbon (δ 213.3). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** showed signals due to six methylene groups [δ 1.88 (4H) and 2.52—3.08 (8H)], four of which are adjacent to an aromatic ring and/or a ketone function, together with signals due to a hydrogen-bonded hydroxy group (δ 7.88), two methoxy groups (δ 3.76 and 3.88), and five protons on the aromatic ring (see Table I). These spectral data are very similar to those of myricanone (**2**), which was isolated at the same time. The ^{1}H-NMR spectrum of **1** showed, together with the signals arising from 15-H, 16-H, and 18-H on the B-ring, a pair of doublets (J=2 Hz) at δ 6.52 and 6.70 instead of the signal (δ 6.61, s) of 19-H in **2** (see Table I). The ^{13}C-NMR spectrum showed five doublets and seven singlets in contrast to that (four doublets and eight singlets) of **2** in the aromatic carbon region in the off-resonance spectrum and signals arising from the carbons on the B-ring [δ 125.3 (C-1), 132.5 (C-14), 132.8 (C-15), 117.1 (C-16), 152.2 or 152.3 (C-17), and 129.4 (C-18)] as in the case of **2**. These observations suggest that **1** has a structure in which a hydroxy group at C-5 in **2** is removed, although another substitution pattern (2,4,5,6-substitution) on the A-ring is still possible. In order to distinguish the two possible substitution patterns, we tried to separate all the signals in the ^{1}H-NMR spectrum. It was found that the spectrum in C_6D_6 solution gave a well-resolved spectrum. Then, we assigned the signals (see Experimental) on the basis of the results obtained from ^{1}H two-dimensional correlated spectroscopy (^{1}H-COSY). Finally, we performed extensive nulcear Overhauser enhancement (NOE) experiments. The results are summarized in Table II. Irradiation at the frequencies of 7-H_2 (δ 2.51) and 4-OMe (δ 3.31) gave NOE's (5.2 and 18.8%,

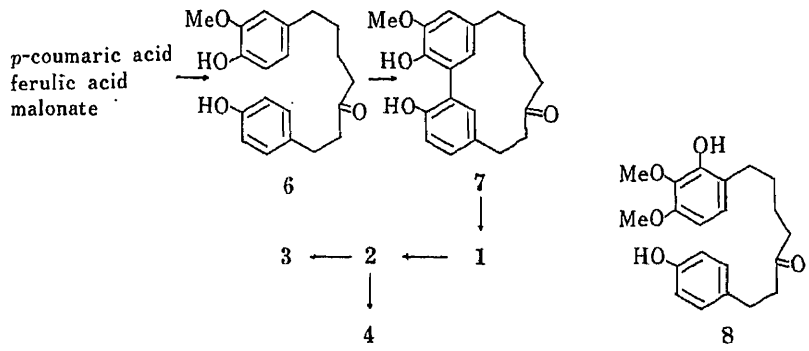


Chart 1. Proposed Biogenetic Pathway

respectively) at 5-H (δ 6.39). NOE's for 19-H (δ 6.56, 13.3%) and 15-H (δ 6.92, 7.4%) were also observed on irradiation at the frequencies of 18-H (δ 7.03) and 13-H_2 (δ 2.92), respectively. The results supported the 2,3,4,6-substitution of the two possible substitution patterns on the A-ring. This assignment was further supported by the following observations. The IR spectrum of **1** (in CCl_4) of a diluted solution showed an absorption band at 3350 cm^{-1} due to 17-OH and no absorption band at 3520 cm^{-1} due to 5-OH as observed in the IR spectrum of **2**.[3,4] In the ^{1}H-COSY spectrum, long-range coupling between the hydrogen-bonded hydroxy proton (δ 8.39) and 15-H was observed *via* zigzag interaction. NOE was observed between the hydroxy group and a methoxy group at C-3 (δ 3.45) in two-dimensional NOE spectroscopy (NOESY). On the basis of these findings, the structure of the new compound was elucidated as **1**, which corresponds to 5-deoxymyricanone. This compound might be a racemic mixture of atropisomers, since **1** and myricanone (**2**) did not show any Cotton effect in the circular dichroism (CD) spectrum, in contrast to myricanol (**4**) [λ_{max} nm (MeOH) ($\Delta\varepsilon$): 299 (−4.01), 258.5 (−5.36) and 238 (−5.04)], although near planarity of the biphenyl portion was suggested from the conjugation band at 251 nm in the UV spectrum.[3]

In the biogenesis of myricanone (**2**) and myricanol (**4**), Begley *et al.*[3] postulated phenolic oxidative coupling at the stage of the 3,4-dimethoxy-5,17-dihydroxy compound (**8**). The isolation of 5-deoxymyricanone (**1**) suggests another possibility, shown in Chart 1. Namely, a diarylheptanoid (**6**) formed *via* condensation of a *p*-coumaric acid, a ferulic acid and a malonate suffers a phenolic oxidative coupling reaction to form compound **7**, which is then methylated to 5-deoxymyricanone (**1**). Hydroxylation at C-5 in **1** gave myricanone (**2**) which was then converted to myricanol (**4**) by reduction of the carbonyl group and to porson (**3**) by introduction of a hydroxy group at C-13 and methylation of the hydroxy group at C-5.

Then, we examined the metabolites of *Pseudomonas syringae* pv. *myricae* py. nov., which causes the bacterial gall of *M. rubra*. As described in Experimental, we isolated five compounds, L-prolyl-L-tyrosine anhydride (**9**), D-prolyl-L-tyrosine anhydride (**10**), L-prolyl-L-valine anhydride (**11**),. L-prolyl-L-phenylalanine anhydride (**12**) and DL-β-phenyllactic acid. These diketopiperazines might be artefacts derived from constituents of the yeast extract and/or peptone, since compounds **9**—**12** were detected clearly on thin layer chromatography (TLC) when the same procedures were applied without microorganisms, although three of these diketopiperazines, **9**, **11** and **12**, were reported as metabolites even when yeast extract and/or peptone were used as constituents of the medium.[6-8] DL-β-Phenyllactic acid was obtained as a crystalline residue, $[\alpha]_D$ 0° (c=2.32), and the structure was confirmed by comparisons of spectral data with those of L-β-phenyllactic acid. L-β-Phenyllactic acid has been isolated as a growth promotor from cultures of *Exobasidium symploci-japonica*[9] and *Gloeosporium laeticolor*.[10] Although our sample is racemic, β-phenyllactic acid could be one of the active substances causing bacterial gall of *M. rubra*.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded with a Hitachi 215 spectrophotometer and UV spectra were taken with a Hitachi 330 spectrophotometer. Unless otherwise noted, ^{1}H- (100 and 400 MHz) and ^{13}C- (50 and 100 MHz) NMR spectra were obtained with JEOL PS-100, JEOL JNM FX-200, and Bruker AM-400 spectrometers, and chemical shifts are given in δ values (ppm) with tetramethylsilane as an internal standard. Optical rotations were measured with a Union Giken PM-201 apparatus. MS were determined with a JEOL DX-300 spectrometer. CD spectra were measured with a JASCO J-500 spectropolarimeter. Kieselgel G (0.05—0.2 mm) was used for column chromatography and Kieselgel GF_{254} precoated plates (0.25 mm) were used for TLC. Preparative layer chromatography was performed on Kieselgel GF_{254} (0.5 mm) or Kieselgel PF_{254} (0.75 mm) plates.

Isolation of Diarylheptanoids from the Gall——Fresh bacterial galls of *Myrica rubra* (2.25 kg) were refluxed with MeOH (3 l) for 30 min and left to stand for 2 months. The MeOH extract was concentrated *in vacuo*. The residue was dissolved in MeOH (600 ml) and the insoluble part was filtered off. Then H_2O (60 ml) was added to the filtrate and the mixture was partitioned with hexane (400 ml × 3). The aqueous MeOH layer was concentrated *in vacuo*. The residue was suspended in H_2O (500 ml) and extracted with AcOEt (500 ml × 3). After being washed with H_2O, the AcOEt extract was dried and evaporated *in vacuo*. The residue (25.24 g) was chromatographed on a silica gel (900 g) column with $CHCl_3$ as an eluant, collecting 500 ml fractions. Fractions 7—8 gave a residue (207 mg) which was separated by preparative layer chromatography ($CHCl_3$–Me_2CO, 9 : 1). The band showing the higher *Rf* value gave **1** (34.0 mg) as an amorphous powder and that showing the lower *Rf* value gave **2** (128 mg) as needles. Fraction 9 gave a residue (822 mg), a portion (414 mg) of which was separated by preparative TLC ($CHCl_3$–Me_2CO, 95 : 5, developed three times) to give **3** (73 mg) and **4** (139 mg). Fractions 10—11 gave a residue (2.37 g) which contained **4** as a main constituent.

5-Deoxymyricanone (**1**): $[\alpha]_D^{22}$ 0° (c=1.03, MeOH). UV λ_{max}^{MeOH} nm (ε): 251 (9476), 295 (5656). IR ν_{max} ($CHCl_3$) cm^{-1}: 3325, 1705, 1590, 1505, 1415, 1270, 1140; ν_{max} (CCl_4 for diluted solution) cm^{-1}: 3350. ^{1}H-NMR ($CDCl_3$) δ: 1.88 (4H), 2.52—3.08 (8H), 3.76 and 3.88 (each 3H, s, 2 × OMe), 6.52 (1H, d, J=2 Hz, 19-H), 6.70 (1H, d, J=2 Hz, 5-H), 6.75 (1H, d, J=3 Hz, 18-H), 6.84 (1H, d, J=8 Hz, 16-H), 7.04 (1H, dd, J=8, 3 Hz, 15-H), 7.88 (1H, OH); (C_6D_6) δ: 1.63 (2H, m, 8-H_2), 1.76 (2H, m, 9-H_2), 2.27 (2H, t, J=7.5 Hz, 10-H_2), 2.31 (2H, m, 12-H_2), 2.51 (2H, m, 7-H_2), 2.92 (2H, m, 13-H_2), 3.31 (3H, s, 4-OMe), 3.45 (3H, s, 3-OMe), 6.39 (1H, d, J=2 Hz, 5-H), 6.56 (1H, d, J=2 Hz, 19-H), 6.92 (1H, dd, J=8, 2.5 Hz, 15-H), 7.03 (1H, d, J=2.5 Hz, 18-H), 7.17 (1H, d, J=8 Hz, 16-H), 8.39 (1H, s, 17-OH). ^{13}C-NMR ($CDCl_3$) δ: 21.9 (t), 25.5 (t), 28.9 (t), 32.4 (t), 42.6 (t), 46.1 (t), 56.1 (q), 61.4 (q), 112. 1 (d), 117.1 (d, C-16), 125.3 (s, C-1), 125.7 (d), 129.4 (d, C-18), 132.1 (s), 132.5 (s, C-14), 132.8 (d, C-15), 136.6 (s), 142.5 (s), 152.2 (s) or 152.3 (s) (C-17), 213.3 (s); (C_6D_6) δ: 22.9 (t, C-9), 25.7 (t, C-8), 29.5 (t, C-13), 32.7 (t, C-7), 42.6 (t, C-12), 46.1 (t, C-10), 55.8 (q, 4-OMe), 61.0 (q, 3-OMe), 112.7 (d, C-5), 117.7 (d, C-16), 126.2 (s), 126.4 (d, C-19), 129.8 (d, C-15), 132.74 (s), 132.78 (s), 133.3 (d, C-18), 136.6 (s), 143.3 (s), 152.9 (s), 153.4 (s), 211.5 (s, C-11). MS *m/z*: 340.1667 $(M)^+$. Calcd for $C_{21}H_{24}O_4$: 340.1674.

Myricanone (**2**): mp 188—191 °C (from $CHCl_3$–MeOH). UV λ_{max} nm (ε): 259 (10383), 296.5 (6675). IR ν_{max} (CCl_4 for diluted solution) cm^{-1}: 3520, 3360. ^{13}C-NMR ($CDCl_3$) δ: 21.9 (t), 24.6 (t), 26.9 (t), 28.9 (t), 42.6 (t), 46.1 (t), 61.3 (q), 61.4 (q), 116.9 (d, C-16), 123.1 (s), 123.2 (s), 125.5 (s), 128.89 (d), 128.94 (d), 132.3 (s, C-14), 132.4 (d, C-15), 138.8 (s), 146.0 (s), 147.8 (s), 151.8 (s, C-17), 213.3 (s). MS *m/z*: 356.1624 $(M)^+$. Calcd for $C_{21}H_{24}O_5$: 356.1624. The identity of **2** was established by direct comparison with an authentic sample.[4]

Porson (**3**): mp 178—180 °C (from diethyl ether). IR ν_{max} ($CHCl_3$) cm^{-1}: 3550, 3325, 1710, 1590, 1505, 1485, 1460, 1405, 1235, 1090, 1005. ^{13}C-NMR ($CDCl_3$) δ: 20.7 (t), 24.7 (t), 28.2 (t), 40.0 (t), 43.0 (t), 60.4 (q), 61.1 (q), 61.9 (q), 77.4 (d), 117.3 (d), 126.5 (s), 126.7 (s), 126.9 (s), 128.8 (d), 130.2 (s), 131.5 (d), 133.1 (d), 145.6 (s), 147.4 (s), 152.4 (s), 153.0 (s), 218.1 (s). MS *m/z*: 386.1718. Calcd for $C_{22}H_{26}O_6$: 386.1729. The identity of **3** was established by comparison of the ^{1}H-NMR spectrum and MS with those reported in the literature.[5]

Myricanol (**4**): $[\alpha]_D^{22}$ −27.6° (c=0.65, $CHCl_3$). MS *m/z*: 358.1776 $(M)^+$. Calcd for $C_{21}H_{26}O_5$: 358,1780. The identity of **4** was established by direct comparison with an authentic sample.[4]

5-Deoxymyricanone Monoacetate (5)——Usual acetylation of **1** (2.5 mg) with a mixture of acetic anhydride and pyridine gave the monoacetate (**5**) (2.8 mg) as a syrup. IR ν_{max} (CCl_4) cm^{-1}: 1760, 1705, 1580, 1500, 1195. ^{1}H-NMR ($CDCl_3$) δ: 1.80 (4H), 2.25 (3H, s, OAc), 2.76 (4H), 3.04 (2H), 3.55 and 3.88 (each 3H, s, 2 × OMe), 6.37 (1H, d, J= 2 Hz, 19-H), 6.70 (1H, d, J=2 Hz, 5-H), 6.78 (1H, d, J=2.5 Hz, 18-H), 7.01 (1H, d, J=8.5 Hz, 16-H), 7.16 (1H, dd, J=8.5, 2.5 Hz, 15-H). MS *m/z*: 382.1758 $(M)^+$. Calcd for $C_{23}H_{26}O_5$: 382.1780.

Isolation of DL-β-Phenyllactic Acid, L-Prolyl-L-tyrosine Anhydride (9), D-Prolyl-L-tyrosine Anhydride (10), L-Prolyl-L-valine Anhydride (11) and L-Prolyl-L-phenylalanine Anhydride (12)——A culture of *Pseudomonas syringae* pv. *myricae* py. nov. maintained on a potato dextrose agar slant (pH 6.8) fortified with 0.5% peptone, 0.005% $Ca(NO_3)_2 \cdot 4H_2O$ and 0.2% $Na_2HPO_4 \cdot 12H_2O$ was used to inoculate four 150 ml aliquots of medium consisting of 2% glucose, 0.5% peptone, 0.3% yeast extract, 0.3% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$ contained in 500 ml Sakaguchi flasks. After inoculation, the flasks were shaken at 29 °C for 24 h. All the culture was used to inoculate 20 l of medium of the same composition supplemented with 0.1% Shinetsu SK 66 as an antifoaming agent in a jarfermenter of 30 l

volume and cultivation was continued for 5 d at 29 °C (agitation speed, 260 rpm; aeration, 17.5 l/min). The mycelia were removed by centrifugation, and the culture filtrate was passed through a column (Amberlite XAD-2, 2 l). The column was eluted with H_2O (10 l), MeOH (4 l) and Me_2CO (3 l), successively. The fractions from MeOH and Me_2CO were combined and evaporated *in vacuo*. The procedures were repeated again. The residues from two fermentations were combined and partitioned between AcOEt and H_2O (pH 3.0). After being washed with sat. NaCl aq. solution, the AcOEt extract was dried and evaporated *in vacuo* to give a residue (9.4 g, residue I). The H_2O layer was extracted with AcOEt after adjustment of the pH to 9 with 1 N NaOH. The AcOEt extract was washed with sat. NaCl aq., dried and evaporated *in vacuo* to give a residue (1.046 g, residue II).

Residue II was chromatographed on silica gel (60 g) with $CHCl_3$–MeOH mixture containing increasing MeOH contents. The eluate with 3—5% MeOH content gave **9** (227 mg) and the eluate with 5% MeOH content gave **10** (57.1 mg).

Residue I was chromatographed on silica gel (300 g) with $CHCl_3$–MeOH as the eluant. The eluate (1.538 g) with 2% MeOH content was purified by repeated chromatography on silica gel with $CHCl_3$–Me_2CO as the eluant to give **11** (171 mg) and **12** (216 mg). The eluate with 4% MeOH content gave a mixture of **9** and **10** (512 mg). The eluate (563 mg) with 4—7% MeOH content was purified by chromatography (silica gel 30 g, $CHCl_3$) to give DL-β-phenyllactic acid (64 mg). The properties of the isolated compounds are as follows:

L-Prolyl-L-tyrosine Anhydride (**9**): mp 144—146 °C, $[\alpha]_D^{28.5}$ −87.3° (c=1.1, MeOH). MS m/z: 260.1143 $(M)^+$. Calcd for $C_{14}H_{16}N_2O_3$: 260.1161. The identity of **9** was established by comparison of the spectral data with those reported in the literature.[11]

D-Prolyl-L-tyrosine Anhydride (**10**): mp 195—197 °C, $[\alpha]_D^{28.5}$ +45° (c=1.6, MeOH). IR ν_{max} (Nujol) cm^{-1}: 3200, 1670, 1655, 1600, 1520. ^{1}H-NMR (CD_3OD) δ: 1.67 (2H, m), 1.93 (1H, m), 2.08 (1H, m), 2.57 (1H, dd, J=10.5, 6.5 Hz), 2.88 (1H, dd, J=14, 4.5 Hz), 3.13 (1H, dd, J=14, 4 Hz), 3.51 (1H, m), 4.17 (1H, t-like, J=4 Hz), 6.74 (2H, d, J=8.5 Hz), 6.98 (2H, d, J=8.5 Hz). ^{13}C-NMR (CD_3OD) δ: 22.4 (t), 29.8 (t), 40.2 (t), 46.1 (t), 59.1 (d), 59.9 (d), 116.3 (d), 126.9 (s), 132.2 (d), 158.1 (s), 167.5 (s), 171.3 (s). MS m/z: 260.1175 $(M)^+$. Calcd for $C_{14}H_{16}N_2O_3$: 260.1161. The identity of **10** was established by direct comparison with an authentic sample prepared according to the literature[6] starting from D-proline and L-tyrosine ethyl ester.

L-Prolyl-L-valine Anhydride (**11**): mp 159—161 °C, $[\alpha]_D^{28.5}$ −128° (c=1.2, MeOH). MS m/z: 196.1207 $(M)^+$. Calcd for $C_{18}H_{16}N_2O_2$: 196.1217. The identity of **11** was established by comparison of the spectral data with those reported in the literature.[7,8]

L-Prolyl-L-phenylalanine Anhydride (**12**): mp 113—115 °C, $[\alpha]_D^{28.5}$ −78.2° (c=1.4, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.76—2.40 (4H, m), 2.76 (1H, dd, J=15, 10 Hz), 3.58 (3H, m), 4.02 (1H, t, J=6 Hz), 4.24 (1H, dd, J=10, 4 Hz), 5.78 (1H, m), 7.30 (5H, m). ^{13}C-NMR ($CDCl_3$ + D_2O) δ: 22.3 (t), 28.2 (t), 36.7 (t), 45.3 (t), 56.1 (d), 59.0 (d), 127.3 (d), 129.0 (d), 129.1 (d), 136.4 (s), 164.9 (s), 169.4 (s). MS m/z: 244.1185 $(M)^+$. Calcd for $C_{14}H_{16}N_2O_2$: 244.1212. The identity of **12** was established by comparison of the IR spectrum with that reported in the literature.[7]

DL-β-Phenyllactic Acid: mp 70—72 °C, $[\alpha]_D^{28}$ 0° (c=2.32, MeOH). MS m/z: 166.0638 $(M)^+$. Calcd for $C_9H_{10}O_3$: 166.0630. The identity of this compound was established by comparison of the spectral data with those of L-β-phenyllactic acid.

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A New Prosapogenin from *Hovenia* Saponin D by Mild Alkaline Degradation

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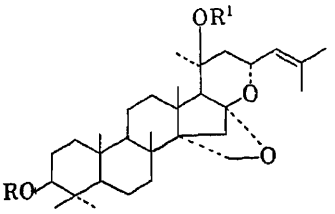
Alkaline cleavage of the glycosidic bonds of saponin D (**2**), obtained from the leaves of *Hovenia dulcis*, gave the genuine sapogenin, jujubogenin (**1**), saponin G (**3**) and a new compound (**4**) whose structure was characterized as 20-*O*-α-L-rhamnopyranosyljujubogenin on the basis of spectral analysis.

Keywords——Rhamnaceae; *Hovenia dulcis*; jujubogenin; 20-*O*-α-L-rhamnopyranosyljujubogenin

In the previous papers[1] we described the novel cleavage of glycosidic bonds of saponins in alcoholic alkali metal solution. This method was considered to be suitable to prepare genuine sapogenins and the corresponding prosapogenins from saponins whose genins are acid-labile, so saponin D obtained from the leaves of *Hovenia dulcis* (Rhamnaceae) was chosen this time for a further study of this cleavage reaction. As described previously,[2] acidic hydrolysis of saponin D did not afford the genuine sapogenin, jujubogenin, but a secondary sapogenin, ebeline lactone. By use of the alkaline cleavage method, the genuine sapogenin and corresponding prosapogenins were prepared from saponin D as follows: saponin D and sodium metal were dissolved in spectroscopic-grade ethanol and the mixture was left at 50 °C for 7 h to give the cleavage products, which were chromatographed on a silica gel column with chloroform and methanol to afford compounds **1**, **2**, **3** and **4**. The three known compounds were identified as jujubogenin (**1**), saponin D (**2**) and saponin G (**3**), respectively, by comparison with authentic samples.[2]

Compound **4** was a white powder, mp 274—276 °C, $[\alpha]_D^{19}$ −42.0 (c=0.37, MeOH). In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **4**, a set of signals assignable to the carbons of jujubogenin and a set of signals attributable to an α-L-rhamnopyranosyl moiety[3] were observed, suggesting **4** to be a rhamnoside of jujubogenin. A comparison of the ^{13}C-NMR spectrum of **4** with that of **1** showed that the C-20 signal of **4** was shifted downfield by 7.2 ppm, while the C-21 and C-22 signals were shifted upfield by 5.7 and 4.2 ppm, respectively, suggesting the rhamnosyl moiety to be linked to the C-20 position.[4] Therefore the structure of **4** was concluded to be 20-*O*-α-L-rhamnopyranosyljujubogenin.



	R	R¹
1	H	H
2	-Glc²—Rham	-Rham
3	-Glc	-Rham
4	H	-Rham

Fig. 1

Table I. ^{13}C-NMR Chemical Shifts

	1	2	3	4		1	2	3	4
1	38.9	38.9	38.6	38.9	C-3				
2	27.9	26.8	26.5	28.2	Glc				
3	78.0	88.7	88.7	77.9	1		105.3	106.6	
4	39.5	39.6	39.6	39.5	2		79.6	75.6	
5	56.0	56.2	56.0	56.0	3		77.7	78.6	
6	18.4	18.8	18.8	18.5	4		72.2	71.8	
7	36.1	35.6	35.6	35.8	5		78.0	78.1	
8	37.6	37.0	37.0	37.5[a)]	6		62.9	63.0	
9	53.0	52.7	52.7	52.9	C-3				
10	37.6	37.0	37.0	37.4[a)]	Rham				
11	21.7	21.5	21.5	21.7	1		101.5		
12	28.6	27.9	28.1	28.6	2		72.2		
13	37.0	37.2	37.2	37.1	3		72.2		
14	53.7	53.7	53.7	53.8	4		74.0		
15	37.0	37.0	37.0	36.7	5		69.4		
16	110.6	109.9	109.8	110.0	6		18.6		
17	53.7	54.8	54.8	54.9	C-20				
18	18.4	18.8	18.8	18.9	Rham				
19	16.3	16.4	16.3	16.4	1		96.3	96.3	96.5
20	68.6	75.7	75.6	75.8	2		72.4	72.6	72.8
21	30.0	24.3	24.3	24.3	3		73.5	73.5	73.7[b)]
22	45.2	40.9	40.9	41.0	4		73.5	73.5	73.6[b)]
23	68.7	68.2	68.2	68.3	5		71.1	71.1	71.3
24	126.8	126.4	126.4	126.6	6		18.6	18.6	18.8
25	134.3	134.6	134.6	134.5					
26	25.8	25.6	25.5	25.6					
27	18.8	19.0	19.1	19.2					
28	28.6	27.9	28.1	28.9					
29	16.3	16.8	16.7	16.4					
30	65.9	65.7	65.7	65.8					

Measured in pyridine-d_5 at room temperature. *a*, *b*) Assignments may be interchanged in each column.

Experimental

The melting points were measured on a Yanagimoto micro melting point hot stage apparatus and are uncorrected. ^{13}C-NMR spectra were recorded on a JEOL FX-100 spectrometer; the chemical shifts are given in ppm (δ) with tetramethylsilane as an internal reference.

Alkaline Cleavage of Saponin D——Saponin D (60 mg) was dissolved in spectroscopic ethanol (7 ml) containing sodium metal (300 mg). The mixture was left at 50 °C for 7 h to cleave the glycoside bonds of saponin D. The reaction mixture was neutralized and concentrated, then the residue was extracted with butanol to give an extract (44.6 mg) containing compounds **1**, **2**, **3** and **4**.

Isolation of Compounds 1, 2, 3 and 4——The butanol extract was chromatographed on a column of silica gel with a mixture of chloroform and methanol (from 25 : 1 to 5 : 1) to afford compounds **1** (3 mg), **4** (7.3 mg), **3** (5.5 mg) and recovered saponin D (**2**) (27 mg).

Identification of the Known Compounds——The known compounds were identified as jujubogenin (**1**), saponin D (**2**) and saponin G (**3**), respectively, on the basis of spectral analysis and comparison of their *Rf* values on thin layer chromatography with those of authentic samples. The ^{13}C-NMR data are given in Table I.

20-*O*-α-L-Rhamnopyranosyljujubogenin (4)——A white powder, mp 274—276 °C, $[\alpha]_D^{19}$ −42.0° (*c* = 0.37, MeOH). *Anal.* Calcd for $C_{36}H_{50}O_8 \cdot H_2O$: C, 68.14; H, 9.15. Found: C, 68.51; H, 9.08. ^{13}C-NMR data are given in Table I.

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Effect of Grifolan on the Ascites Form of Sarcoma 180

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The antitumor activity of a (1→3)-β-D-glucan, grifolan, which was obtained from a fungus, *Grifola frondosa*, on the ascites form of Sarcoma 180 in ICR mice was examined. Grifolan was effective on the ascites tumor when it was administered before tumor transplantation (days −7—−1) as a single dose of 2 mg/mouse by i.p. injection, but administration after the tumor transplantation was ineffective. The antitumor activity was transferred to untreated mice by injecting the peritoneal exudate cells obtained from grifolan-treated mice. The antitumor activity of grifolan was augmented by vaccination of mice with live sarcoma 180 cells (s.c. injection).

Keywords——*Grifola frondosa*; grifolan; ascite tumor; sarcoma 180; glucan; antitumor activity

(1→3)-β-D-Glucan is now clinically used as an immunomodulating anticancer drug, and basic and clinical examinations of the glucan are actively progressing.[1] Our group has obtained an antitumor glucan, grifolan, from the fruit body, matted mycelium, and liquid culture of a fungus, *Grifola frondosa*, and has been studying the physicochemical properties and antitumor mechanisms.[2] During these studies, we found that grifolan increased the number of peritoneal exudate cells (PEC) when given by i.p. injection,[2a] and that phagocytic cells are important for its antitumor activity because the activity was lowered by injecting carrageenan.[2b] It is known that several immunomodulating materials induce antitumor activity mediated by PEC.[3] In a preliminary study, PEC from grifolan-injected mice showed antitumor activity *in vitro*. While glucans are known to be less effective against ascites tumor,[4] a certain effect would be expected because the number of PEC was increased and PEC showed antitumor activity *in vitro* after the injection of grifolan.[2a,b] Thus, it was of interest to examine the activity of grifolan against ascites tumor. In this paper, we describe the effect of grifolan on ascites form of sarcoma 180.

Materials and Methods

Preparation of Grifolan——Grifolan was purified from NMF-5, which was obtained from the matted mycelium of *Grifola frondosa* by alkaline extraction, by diethylaminoethyl (DEAE)-Sephadex A-25 chromatography and ethanol precipitation.[2c,d]

Evaluation of Antitumor Activity——Male ICR mice, 6 weeks old, weighing 27—30 g were obtained from Shizuoka Cooperative Association for Laboratory Animals, Hamamatsu. Sarcoma 180 tumor cells (ascites form) were kindly supplied by Dr. T. Sasaki, the National Cancer Center Research Institute, Japan, and were maintained by weekly passage in ICR mice. Antitumor activity was evaluated by comparing the survival (days) with that of control groups, and is given as the mean survival time ±S.D., median survival time (T/C), and number of cured mice at the indicated time. The significance was evaluated by means of the generalized Wilcoxson test, and $p<0.05$ was taken as the criterion of a significant difference.

Results

Effect of Timing of Grifolan Treatment on Antitumor Activity against Ascites Form of Sarcoma 180

Grifolan (2 mg/mouse) was injected into ICR mice intraperitoneally at days −7 to +7. Sarcoma 180 (1×10^6 or 1×10^7 cells) was intraperitoneally transplanted at day 0, and the survival times of mice in each group were compared. As shown in Table I, the groups given grifolan after the tumor transplantation showed little or no prolongation of survival as compared with the control group (1×10^7 tumor cells, day 7). On the other hand, survival in the preadministered groups (especially days −7, −5, and −3) was significantly prolonged, though only in the groups given 1×10^6 tumor cells. Thus, it is suggested that grifolan is effective against the ascites form of tumor, especially when injected at days −7——3.

Dose Dependence of Grifolan Antitumor Activity against Ascites Form of Sarcoma 180

To determine the optimum dose of grifolan for exhibiting antitumor activity against ascites tumor, 100, 500, or 2000 μg of grifolan was injected at various times, and the survival times were compared (Table II). Although the 100 or 500 μg groups showed statistically significant effects, these groups showed only little prolongation of survival (T/C less than 200) and few survivors. Therefore, it is suggested that the optimum dose of grifolan is 2 mg/mouse or more.

Antitumor Activity of Peritoneal Exudate Cells from Grifolan-Treated Mice against Ascites Form of Sarcoma 180

Previous work has suggested that grifolan shows no direct cytocidal activity against tumor cells.[2f-h] Similarly, activation of the immune system also appears to be involved in the antitumor action of grifolan against the ascites tumor. To examine the involvement of peritoneal exudate cells (PEC) in the antitumor activity of grifolan, grifolan-induced PEC were recovered and transferred into the peritoneal cavity of mice with sarcoma 180 cells.

Table I. Effect of Administration Timing on the Antitumor Activity of Grifolan against Ascites Form of Sarcoma 180[a]

Day	Mean survival time (d, mean ± S.D.)[b]	Median survival time (d)	T/C[c] (%)	66-d survivors/ total[d]
(1×10^6 cells)				
−7	36.0 ± 20.0[e]	39	229	2/10
−5	46.2 ± 25.4[f]	>66	>388	5/10
−3	36.4 ± 21.8[g]	32	188	3/10
−1	23.9 ± 15.7[g]	19	112	1/10
+1	13.2 ± 1.2	13	76	0/10
+3	14.1 ± 2.3	13.5	79	0/11
+5	21.4 ± 16.4	14.5	85	1/10
+7	15.6 ± 1.8	15	88	0/10
Nil	15.5 ± 3.9	17	100	0/10
(1×10^7 cells)				
−7	15.3 ± 4.4	15	111	0/10
−3	14.5 ± 2.7	15	111	0/10
+3	17.3 ± 17.1	12	80	1/10
+7	25.4 ± 21.6[g]	16.5	122	2/10
Nil	13.3 ± 2.5	13.5	100	0/10

a) Sarcoma 180 cells (1×10^6 cells (upper) or 1×10^7 cells (lower)) were transplanted intraperitoneally on day 0. *b*) Survival of 67 d was assumed for mice still alive on day 66. *e*) $p<0.01$; *f*) $p<0.02$; *g*) $p<0.05$. *c*) The median survival time of treated groups (T) *vs.* the control group (C). *d*) The ratio of 66-d survivors to total mice.

TABLE II. Effect of Various Doses of Grifolan on Ascites Form of Sarcoma 180[a]

Dose (μg)	Days	Mean survival time (d, mean ± S.D.)[b]	Median survival time (d)	T/C[c] (%)	73-d survivors/ total[d]
(5×10^5 cells)					
100	−7	17.6 ± 4.9[e]	17	142	0/17
	−4	21.4 ± 8.1[g]	18.5	154	0/10
	−1	16.5 ± 3.3	17	142	0/10
500	−7	17.0 ± 8.1	16.5	138	0/10
	−4	17.5 ± 3.3[f]	18	150	0/10
	−1	18.9 ± 7.4[e]	18.5	154	0/10
2000	−7	54.1 ± 24.5[h]	>73	>608	6/10
	−4	57.2 ± 20.5[h]	>73	>608	6/10
	−1	39.5 ± 20.7[h]	32.5	271	2/11
Nil		12.9 ± 3.9	12	100	0/10
(1×10^6 cells)					
100	−7	14.7 ± 2.7	14.5	100	0/10
	−4	17.7 ± 1.7	18	124	0/10
	−1	15.6 ± 2.9	15.5	107	0/10
500	−7	15.3 ± 5.0	17	117	0/10
	−4	27.3 ± 24.2	17	117	2/10
	−1	17.9 ± 2.2	18.5	128	0/10
2000	−7	40.1 ± 28.3[f]	23	159	3/10
	−4	65.8 ± 12.1[h]	>73	>503	6/10
	−1	19.5 ± 4.0[f]	19.5	134	0/10
Nil		14.4 ± 4.1	14.5	100	0/10

a) Sarcoma 180 cells (5×10^5 cells (upper) or 1×10^6 cells (lower) were transplanted intraperitoneally on day 0. *b*) Survival of 74 d was assumed for mice still alive on day 73. *e*) $p < 0.05$; *f*) $p < 0.02$; *g*) $p < 0.01$; *h*) $p < 0.002$. *c*) The median survival time of treated groups (T) *vs.* the control group (C). *d*) The ratio of 73-d survivors to total mice.

Grifolan (2 mg/mouse) was injected i.p. at day −3, and 0.9×10^7 PEC/mouse were induced (day 0). Thus, 1×10^7 PEC were mixed with sarcoma 180 cells (5×10^5 cells) and transplanted intraperitoneally into each mouse (11 mice). The survival time of the control group, in which mice received 5×10^5 sarcoma 180 cells intraperitoneally at day 0, was 12.4 ± 2.6 d, and all the mice (11 mice) died within 17 d. On the other hand, survival of the PEC-administered group was prolonged (18.5 ± 9.1 d) and there was 1 survivor at day 43 ($p < 0.05$). It is suggested that PEC cells are involved, at least in part, in the antitumor activity against ascites tumor. It is not yet known whether normal peritoneal cells show a similar effect, but in any case, the overall effect would be less because the numbers of cells would be about 10 times less than in the grifolan-administered group.[2a,b]

Effect of Immunization of Mice with Sarcoma 180 Cells on Antitumor Activity of Grifolan against Ascites Form of Sarcoma 180

As described above, grifolan was effective against ascites tumor under limited conditions. Since combination therapy, such as chemotherapy, surgery, or vaccination, might augment the effects of grifolan, we tried to use live sarcoma 180 vaccine. Sarcoma 180 cells were administered subcutaneously at day −7. At days −4, −1, +1, grifolan (2 mg/mouse) was administered intraperitoneally. Sarcoma 180 cells were administered intraperitoneally at day 0. As shown in Table III, all mice which carried ascites form of sarcoma 180 died without grifolan treatment. Grifolan-treated groups showed prolonged survival, and almost all the mice were cured. In contrast to the results without vaccination, the number of survivors increased and administration at day 1 was also effective in the case of vaccination. Further,

TABLE III. Augmentation of Antitumor Activity of Grifolan against Ascites Form of Sarcoma 180 by s.c. Injection of S-180[a]

Day	Mean survival time (d, mean ± S.D.)[b]	Median survival time (d)	T/C[c] (%)	35-d survivors/ total[d]
−4	34.4 ± 1.8[e]	>35	>228	7/8
−1	32.6 ± 6.7[f]	>35	>216	7/8
1	32.6 ± 6.7[f]	>35	>216	7/8
Nil	15.1 ± 4.6	18	100	0/8

a) Sarcoma 180 cells (5×10^6 cells) were transplanted subcutaneously on day −7 and intraperitoneally on day 0. Grifolan (2 mg/mouse) was intraperitoneally injected at the indicated time. *b*) Survival of 36 d was assumed for mice still alive on day 35. *c*) The median survival time of treated groups (T) *vs.* the control group (C). *d*) The ratio of 35-d survivors to total mice. *e*) $p < 0.002$; *f*) $p < 0.01$.

the treatment was effective even against 5×10^6 sarcoma 180 cells.

Though the data are limited, combination therapy appears to augment the antitumor activity of grifolan against ascites tumors.

Discussion

Previously, we showed that grifolan does not act directly against tumor cells, but activates certain immune systems.[2f] In the solid tumor, treatment of mice with carrageenan before or at tumor transplantation suppressed the antitumor activity of grifolan, but treatment after that did not.[2b] From these findings, it is suggested that the phagocytic system is important not at the degradation stage of sarcoma 180 but at the tumor recognition stage. Further, in assays with syngenic systems *in vitro*, grifolan activated natural killer cells, cytocidal macrophages, and cytotoxic T cells (to be published). These findings suggested that grifolan activates several effector mechanisms of antitumor immunity. It is not yet clear what is the most important event in the induction of antitumor activity by grifolan. As described previously, grifolan is effective against the solid form of sarcoma 180 even at the dose of 100 μg/mouse, both before and after the transplantation of sarcoma 180.[2f] However, in the case of ascites tumor, it is only effective at more than 2000 μg/mouse and before the transplantation of sarcoma 180. The differences of optimum dose for activity against solid and ascites tumors suggest that different effector mechanisms are mainly involved in the actions against solid and ascites tumors. The PEC induced by grifolan possessed antitumor activity, suggesting the involvement of immune systems in the antitumor activity against the ascites tumor. Precise characterization of the immune mechanisms involved is in progress.

Several antitumor glucans are now used clinically, and are usually used in combination with chemotherapy. In experimental murine tumor systems, some glucans are effective against ascites tumor when administered with chemotherapeutic drugs.[4] The polysaccharide EA was reported to be effective against tumors when combined with cryosurgery or vaccination.[5] In the case of grifolan, antitumor activity against ascites tumor was observable only under limited conditions. However, as shown in this paper, the antitumor activity of grifolan against ascites tumor was augmented by vaccination. Thus, it appears that grifolan should act more effectively against various types of tumors when used with various combination therapies.

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Effect of Calcium-Binding Protein on the Activation of Phosphorylase *a* in Rat Hepatic Particulate Glycogen by Ca^{2+}

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The effect of a calcium-binding protein (CaBP) isolated from rat liver cytosol on phosphorylase *a* activity in the hepatic particulate glycogen was investigated. The particulate glycogen phosphorylase *a* activity was significantly increased by addition of Ca^{2+} in the range of 1.0—10^3 μM. This increase was not prevented by the presence of *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7 100 μM), an inhibitor of calmodulin. The elevation of phosphorylase *a* activity by 10 μM Ca^{2+} addition was significantly inhibited by the presence of CaBP at a concentration of greater than 3.0 μM. This inhibition was complete at 7.0 μM CaBP. CaBP itself had no effect on the enzyme activity. Of various metals (20 μM) used, addition of Zn^{2+} caused a significant decrease of the particulate glycogen phosphorylase *a* activity, while Mg^{2+}, Mn^{2+} and Cd^{2+} had no effect on the enzyme activity. The presence of 3.5 μM CaBP did not block the inhibition of the enzyme activity by Zn^{2+} addition, indicating that CaBP uniquely reverses the Ca^{2+} effect. The present study suggests that CaBP regulates the effect of Ca^{2+} on phosphorylase *a* activity in the hepatic particulate glycogen of rats.

Keywords——calcium-binding protein; calcium ion; zinc; phosphorylase *a*; rat liver glycogen

Introduction

It is well known that Ca^{2+} plays an important role in the regulation of many cell functions.[1,2] The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations.[3,4] The Ca^{2+} effect is amplified through calmodulin in liver cells.[1,2] More recently, it was found that a novel calcium-binding protein (CaBP) isolated from rat liver cytosol reverses the activating effect of Ca^{2+}-calmodulin on the liver cytosolic enzyme.[5] CaBP may regulate Ca^{2+} action in liver cells.

Phosphorylase *a* in the hepatic particulate glycogen fraction of rats may be a Ca^{2+}-sensitive enzyme.[6] Therefore, the present study was undertaken to clarify whether Ca^{2+} increases the activity of hepatic particulate glycogen phosphorylase *a* and whether the Ca^{2+} effect is regulated by CaBP. The present results demonstrate that CaBP reverses the enzyme activation by Ca^{2+}. This further supports the view that CaBP can regulate Ca^{2+} action on liver cell functions.

Materials and Methods

Animals——Male Wistar rats, weighing 100—130 g were used. They were obtained commercially (Nippon Bio Supply Center, Tokyo, Japan). The animals were given commercial laboratory chow containing 1.1% Ca, 1.1% P and 57.4% carbohydrate (Oriental Test Diet, Tokyo, Japan) and tap water freely.

Isolation of CaBP——CaBP in the cytosol fraction of rat liver was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously.[7]

Preparation of Hepatic Particulate Glycogen——Particulate glycogen was prepared by a modification of the

method of Tata.[8] The liver was perfused with 0.44 M sucrose solution containing 60 mM NaF, frozen immediately, then cut into small pieces, which were suspended in ice-cold 0.44 M sucrose solution containing 60 mM NaF (1 g of tissue to 9 ml of medium) and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. Particulate glycogen was separated from the mitochondria-free supernatant by centrifugation at 80000×*g* for 90 min. The glycogen pellet containing the enzyme was suspended in ice-cold distilled water.

Analytical Methods——Phosphorylase *a* assay was carried out under optimal conditions.[9] The usual reaction mixture contained 12 mM glucose-1-phosphate, 0.35% glycogen, 30 mM NaF, 200 mM Tris-HCl buffer (pH 6.7), and particulate glycogen (containing 75—85 μg as protein), in a final volume of 1.0 ml. In some experiments, the test system contained calcium chloride (10^{-5}—10^{3} μM Ca^{2+}) and/or CaBP (1.9—15 μM) in the above assay system. Assays were conducted by incubation for 10 min at 37 °C, and the reaction was stopped by addition of 1.0 ml of 5% trichloroacetic acid, then the precipitate was removed by centrifugation. The inorganic phosphate in the supernatant was determined according to the method of Nakamura and Mori.[10] The enzyme activity was expressed as nmol of inorganic phosphate released per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[11] All other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Statistical Methods——The significance of differences between values was estimated by using Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

The effect of Ca^{2+} addition on phosphorylase *a* activity in the particulate glycogen prepared from rat liver is shown in Fig. 1. The particulate glycogen phosphorylase *a* activity was significantly increased by addition of Ca^{2+} in the range of 1.0—10^{3} μM. As the concentration of Ca^{2+} was increased, the increase in phosphorylase *a* activity became greater. This increase was not inhibited by the presence of 100 μM W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), an inhibitor of calmodulin (data not shown), suggesting that the particulate glycogen phosphorylase *a* is not activated by Ca^{2+}-calmodulin.

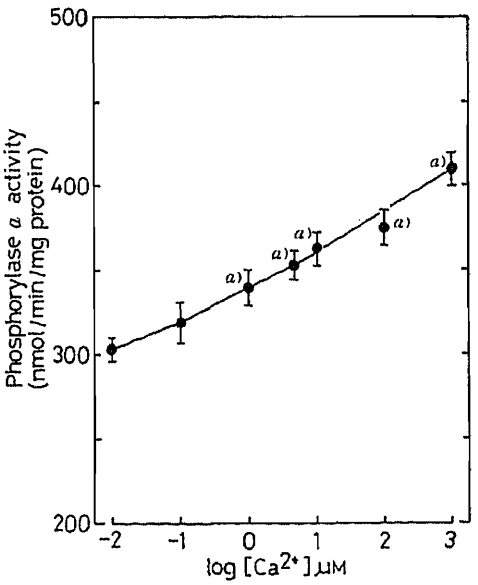

Fig. 1. Effect of Increasing Concentrations of Ca^{2+} on the Activity of Phosphorylase *a* in the Hepatic Particulate Glycogen of Rats

Addition of 10^{-5} or 10^{-4} μM Ca^{2+} did not alter the enzyme activity. Each value represents the mean ± S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the value without Ca^{2+}.

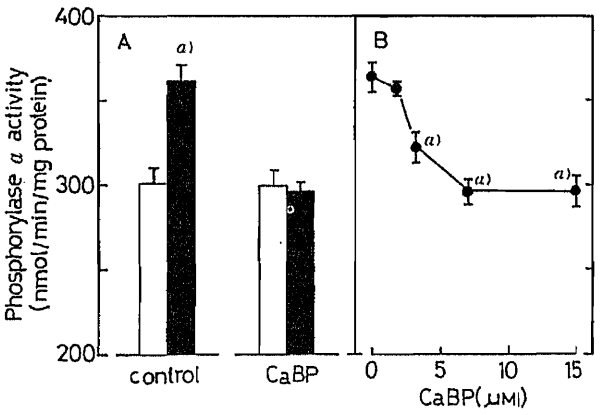

Fig. 2. Effect of CaBP on the Increase in Phosphorylase *a* Activity of the Hepatic Particulate Glycogen Caused by Ca^{2+} Addition

Figure A shows the effect of CaBP (7.0 μM) on the increase of phosphorylase *a* activity caused by 10 μM Ca^{2+} addition. Figure B shows the effect of increasing concentrations of CaBP (1.9, 3.0, 7.0 and 15 μM). Each value represents the mean ± S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the control value. □, control; ■, 10 μM Ca^{2+} addition.

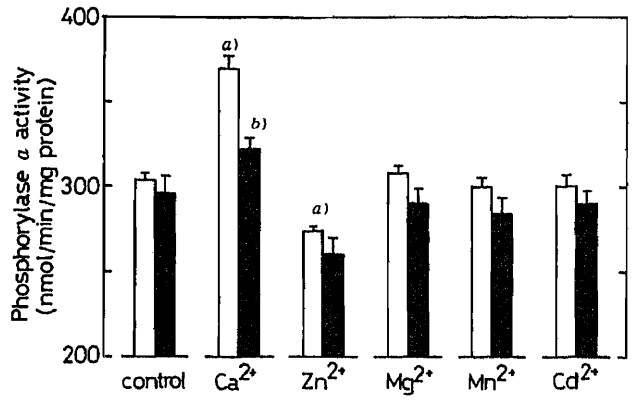

Fig. 3. Effect of Various Metals on Phosphorylase *a* Activity in the Hepatic Particulate Glycogen of Rats

The enzyme activity was measured in the reaction mixture containing 20 μM metal (final concentration) in the presence or absence of 3.5 μM CaBP. Each value represents the mean ± S.E.M. of 4 experiments. *a*) $p<0.01$, as compared with the control (none) value. *b*) $p<0.01$, as compared with the metal (none) value. □, none; ■, CaBP.

The effect of a CaBP isolated from the cytosol of rat liver on the increase of phosphorylase *a* activity in the hepatic particulate glycogen caused by addition of Ca^{2+} (10 μM) was examined, and the results are shown in Fig. 2. The increase in the enzyme activity caused by Ca^{2+} addition was reversed to the control level by the presence of CaBP (7.0 μM) (Fig. 2A). CaBP itself did not have a significant effect on the enzyme activity of the control, although it caused a slight decrease of the activity. This supports the view that the calcium added to the reaction mixture was bound to CaBP, because this protein has 6—7 high-affinity binding sites per molecule of protein.[12] The inhibitory effect of CaBP on the increase in the hepatic particulate glycogen phosphorylase *a* activity caused by 10 μM Ca^{2+} addition was apparent in the presence of 3.5 μM CaBP, and the effect was saturated at the concentration of 7.0 μM (Fig. 2B).

The effect of various metals on phosphorylase *a* activity in the hepatic particulate glycogen is shown in Fig. 3. The enzyme activity was significantly decreased by addition of 20 μM Zn^{2+}, while Mg^{2+}, Mn^{2+} and Cd^{2+} at a concentration of 20 μM had no effect on the enzyme activity. The inhibitory effect of Zn^{2+} on phosphorylase *a* activity was not reversed by the presence of 3.5 μM CaBP, although the effect of Ca^{2+} was blocked by CaBP. CaBP had a unique effect on Ca^{2+}.

Discussion

The activity of phosphorylase *a* in the hepatic particulate fraction of rats was significantly increased by addition of Ca^{2+} in the range of 1.0—10^3 μM. This increase, however, was not inhibited by a calmodulin inhibitor (W-7), suggesting that the increase in the particulate glycogen phosphorylase *a* activity by Ca^{2+} is independent of calmodulin. Ca^{2+} may directly activate phosphorylase *a* in the hepatic particulate glycogen of rats. The particulate glycogen phosphorylase *a* activity was not increased by other metal ions; Ca^{2+}, of the metal ions used, could uniquely increase the particulate glycogen phosphorylase *a* activity.

The ability of Ca^{2+} to increase phosphorylase *a* activity in the hepatic particulate glycogen was blocked by the presence of a CaBP isolated from rat liver cytosol. This effect of CaBP was appreciable at the concentration of 3.0 μM, and was complete at 7.0 μM. The previous investigation on the role of CaBP in liver cell functions demonstrated that the CaBP at a lower concentration can reverse the activation of liver cytosol fructose-1,6-diphosphatase by Ca^{2+}-calmodulin.[5] CaBP at 0.7 μM completely reversed the activation of fructose-1,6-diphosphatase by Ca^{2+}-calmodulin.[5] Also, the activation of liver cytosolic pyruvate kinase by Ca^{2+}, independently of calmodulin, was completely blocked by 0.7 μM CaBP.[13] From these investigations, it is assumed that the liver cytosolic enzyme is more sensitive than the liver particulate enzyme to the action of CaBP to reverse the activation

of enzymes by Ca^{2+} and/or calmodulin.

Of various metal ions at a comparatively low concentration, Zn^{2+} caused a significant decrease of phosphorylase *a* activity in the hepatic particulate glycogen. This decrease was not reversed by the presence of CaBP, whereas the protein blocked the effect of Ca^{2+} on the enzyme. This result may suggest an important role of Ca^{2+}, among the metals tested, in the stimulation of hepatic glycogenolysis, and indicates a specific regulatory action of CaBP on the Ca^{2+} effect. The molecular weight of CaBP was estimated to be 28800, and the Ca^{2+} binding constant was found to be $4.19 \times 10^5\,M^{-1}$ by equilibrium dialysis.[12] This protein has 6—7 high-affinity binding sites per molecule of protein. Thus, CaBP differs from calmodulin, which modulates many biochemical effects of Ca^{2+} in cells.[1,2] CaBP may play a different role from calmodulin in the regulation of liver cell functions by Ca^{2+}. At present, the mechanism of reversal of the Ca^{2+} effect on enzymes is unknown. It is possible, however, that CaBP binds Ca^{2+} and thus weakens the effect of the metal.

Since phosphorylase *a* is activated by Ca^{2+} and this activation is regulated by CaBP, this regulatory system may have a cell-physiological significance. Phosphorylase *a* in hepatic particulate glycogen causes the breakdown of glycogen. An increase of Ca^{2+} in hepatic cytosol following hormonal stimulation directly activates phosphorylase *a* and may stimulate glycogen breakdown. Presumably, CaBP located in hepatic cytosol can prevent a progressive breakdown of glycogen stimulated by Ca^{2+}, because the CaBP can reverse phosphorylase *a* activation by the metal.

The previous[5,13] and present investigations demonstrate that a CaBP isolated from rat liver cytosol, can reverse the activation of hepatic enzymes by Ca^{2+} and/or calmodulin. CaBP probably plays a role in regulation of Ca^{2+} action on liver cell function.

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Conformation of Grifolan in the Fruit Body of *Grifola frondosa* Assessed by Carbon-13 Cross Polarization–Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy

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Grifola frondosa produces an antitumor (1→3)-β-D-glucan, grifolan, which possesses two kinds of conformations in the solid state, *i.e.*, helix (curdlan type) and native (laminaran type) (Ohno *et al.*, *Chem. Pharm. Bull.*, **34**, 2555 (1986)). In this paper, the glucan conformation in the fruit body was examined by using carbon-13 cross polarization–magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy. Before analysis, the fruit body was treated as follows: amylase digestion, amylase digestion-periodate-borohydride treatment, hot water extraction, cold alkali extraction, or hot alkali extraction. Except for the cold and the hot alkali-extracted fruit bodies, all preparations showed C-3 signals at about 86 ppm, resembling those of the native form. These findings suggested that the (1→3)-β-D-glucan conformation in the fruit body is the "native form."

Keywords——cross polarization–magic angle spinning-NMR (CP/MAS-NMR); glucan; grifolan; *Grifola frondosa*; antitumor glucan; (1→3)-β-D-glucan

The ultrastructure of (1→3)-β-D-glucans is thought to be important for their antitumor activity. Induction of antitumor activity by the urea treatment of pachyman (U-pachyman) is one reason for this view.[1] In the case of schizophyllan, high temperature treatment (150 °C) at a low glucan concentration degraded the helical conformation into small aggregates,[2] and reduced the antitumor activity. Thus, triple helical structure is thought to be one of the essential structural requirements for exhibiting antitumor activity in the case of schizophyllan. Zymolyase is an endo-(1→3)-β-D-glucanase, produced by *Arthrobacter luteus*.[3] Changes in the conformation of glucans also affected their digestivity with zymolyase; for example, boiling treatment of pachyman increased the digestivity.[3]

The physical, chemical, and physicochemical properties of the antitumor (1→3)-β-D-glucans, such as curdlan, lentinan, and schizophyllan, have been extensively studied. The X-ray crystallography of curdlan, lentinan, scleroglucan, and schizophyllan strongly suggested the existence of triple helix structure.[4] Saito *et al.* have applied cross polarization–magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy to determine the conformations of these glucans, and suggested that the chemical shift of C-3 is strongly affected by the torsion angles.[5] It is also indicated that (1→3)-β-D-glucans possess at least 4 types of solid-state conformations: form I (curdlan-type), form II (laminaran-type), form III (laminaripentaose-type), and form IV (dimethylsulfoxide-adduct). Recently, we have studied the conformations of (1→3)-β-D-glucan fractions obtained by various preparation procedures from the fungus *Grifola frondosa*, and suggested that two kinds of conformations, native and helix, were present.[6] It is also suggested that, in the liquid culture, the glucans (LLFD, LELFD) were synthesized in the native form and transferred into the helix form during the purification by treatment with urea and/or sodium hydroxide. By comparison with the spectra

reported by Saito *et al.*, it appears that the helix form correspond to form I (curdlan type) and the native form to form II (laminaran type).

Fungal extracts are used as traditional drugs for cancer therapy. Recently, antitumor activity was also observed on feeding of mushroom powder.[7] It would be interesting to know the conformation of glucans in the fungal fruit body and what changes occur during extraction and digestion. In this study, we examined the conformation of (1→3)-β-D-glucan in the fruit body of *G. frondosa* by CP/MAS NMR spectroscopy. The occurrence of conformational rearrangement during extraction is discussed.

Materials and Methods

Materials——The fruit body of *Grifola frondosa* was cultured in bottles by Nippon Beet Sugar Mfg. Co., Ltd.[8] Amylase-digested and periodate-borohydride-treated fruit bodies were prepared as described previously.[9] The hot water extract, cold alkali extract, and hot alkali extract were prepared as described previously.[10] LELFD, LLFD, and grifolan were prepared and purified as described previously.[11]

Carbon-13 CP/MAS NMR Spectra——^{13}C-CP/MAS NMR spectra were measured with a JEOL-FX 200 instrument, equipped with a CP/MAS unit operating with JEOL CP/MAS software. The spectra were measured with the use of a Dyflon rotor. Contact time, pulse interval, and number of pulses were 1 ms, 1—2 s, and 500—2000, respectively. Chemical shifts relative to tetramethylsilane (TMS) were determined by using adamantane signals (29.5 ppm).

Results and Discussion

From the results of chemical characterization of several extracts from the fruit body of *G. frondosa*, major carbohydrate components of the fruit body were found to be (1→4)-α-, (1→3)-β-, and (1→6)-β-D-glucans.[10,12] To increase the relative content of (1→3)-β-D-glucan in the fruit body, glucoamylase digestion of the fruit body was performed (A-G.f.).[9] Carbon-13 NMR spectral analysis of the fruit body as an aqueous suspension and the chemical examination of the hot water extract (AHW) indicated a decreased content of (1→4)-α-D-glucan in the fruit body.[9] Thus, the major glucan components of A-G.f. are thought to be (1→3)-β- and (1→6)-β-. Figure 1a shows the CP/MAS spectrum of A-G.f. By comparison with the spectra of curdlan (Fig. 1g) and LELFD (Fig. 1f), it was clear that the C-3 ^{13}C-NMR signal (85 ppm) appeared at a quite similar position to that of LELFD. This suggested that the conformation of (1→3)-β-D-glucan before extraction was not "helix" but "native." The fruit body contains (1→6)-β-D-glucan and chitin as well as (1→3)-β-D-glucan. It is thus possible that the C-3 signal overlap with signals of other glycans. To degrade the (1→6)-β-D-glucan, periodate oxidation and borohydride reduction of the fruit body were performed (I/B-A-G.f.).[9] Figure 1b shows the CP/MAS spectrum of I/B-A-G.f. The spectrum around the C-3 signal was not much altered from that of A-G.f. Signals of chitin would not appear because of the low content of chitin, as described below. Therefore, it is suggested that the conformation of (1→3)-β-D-glucan in the fruit body is "native."

To examine the conformation of (1→3)-β-D-glucan in the fruit body more precisely, the fruit body was extracted successively with hot water (autoclaving), cold alkali (10% NaOH–5% urea, 4 °C), and hot alkali (10% NaOH–5% urea, 65 °C)[10] and then the CP/MAS spectra of the residues (HWR, CAR, HAR, respectively) were measured. As shown in the previous papers, HWE contained a large proportion of (1→4)-α-glucan and a small proportion of (1→3) and (1→6)-β-glucans.[9,10] On the other hand, CAE and HAE contained a large proportion of (1→3)-β-glucan.[10] CP/MAS spectra of the residues are shown in Fig. 1. The intensities of the signals at 23, 56, and 174 ppm were increased during the extraction, and the spectrum of HAR was similar to that of chitin (Fig. 1h). It is suggested that the major carbohydrate component of HAR was chitin, and that the intensity of signal at 56 ppm is related to the relative content of chitin in preparations. Thus, signals attributable to chitin

would appear only as noise in the HWR and CAR fractions, and the signals at around the C-3 region would be attributable to (1→3)-β-D-glucans. When the spectra of HWR and CAR are compared with that of A-I/B-G.f., only a broad C-3 signal can be seen. However, chemical characterization of CAE and HAE (those contained components of HWR and CAR, respectively) indicated that both residues contained large amounts of (1→3)-β-D-glucan.[10] Further, the ^{13}C-NMR spectra of CAR and HAR as aqueous suspensions showed signals attributable to (1→3)-β-D-glucan.[10] It is suggested that the torsion angles of (1→3)-β-D-glucans in HWR and CAR are more varied than those of LLFD and LELFD, which are (1→3)-β-D-glucans produced extracellularly by liquid cultivation of *G. frondosa*. The differences of the CP/MAS spectra of A-G.f. and HWR and/or CAR could result, in part, from the treatment of the mushroom under various drastic conditions, such as heat, sodium hydroxide and urea, because such conditions would induce denaturation of the macromolecular components.[13] In particular, alkali and urea treatments induce conformational change of the glucan in solution.[13] However, it is also possible that the biosynthetic mechanism of (1→3)-β-D-glucans in the mushroom is more complicated than in liquid culture, and variability of torsion angles might arise biosynthetically. This possibility is supported by the fact that the degree of branching of the (1→3)-β-D-glucan in the mushroom *Lentinus edodes* varies in relation to the location of each glucan in the mushroom.[14]

It is concluded that the (1→3)-β-D-glucan conformation in the fungal fruit body is "native form." Previously, we showed that the conformation of the (1→3)-β-D-glucan obtained from the liquid cultured broth of *G. frondosa* was also "native." However, the conformation of curdlan, which is a linear (1→3)-β-D-glucan obtained from *Alcaligenes faecalis*, is known to be "helix." This difference may be dependent on the biosynthetic route, but further work is necessary to establish this. Hot water extracts of several mushrooms have been used traditionally as anticancer drugs. Feeding of the mushroom was also shown to be

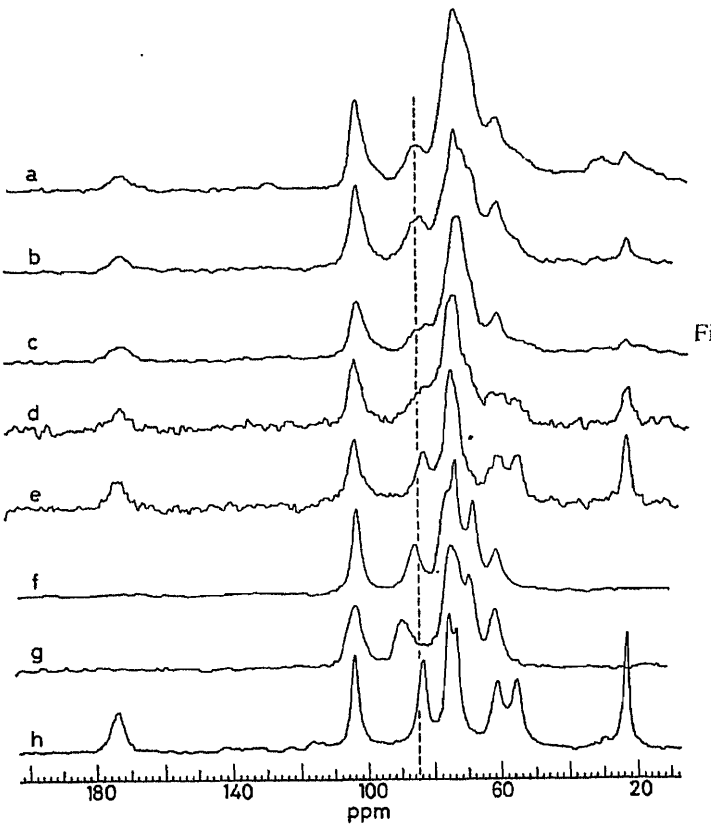

Fig. 1. Carbon-13 CP/MAS NMR Spectra of the Fruit Body of *Grifola frondosa*

a, Amylase-treated *G. frondosa* (A-G.f.); b, periodate-oxidized and then borohydride-reduced A-G.f. (A-I/B-G.f.); c, hot water-extracted residue of *G. frondosa* (HWR); d, cold alkali-extracted residue of *G. frondosa* (CAR); e, hot alkali-extracted residue of *G. frondosa* (HAR); f, extracellular polysaccharide from liquid cultured *G. frondosa* (LELFD, native form); g, curdlan (helix form); h, chitin. Chemical shifts of the carbon signals of the reference compounds were as follows. LELFD: C-1, 103.4 ppm; C-2, 74.2 ppm; C-3, 86.3 ppm; C-4, 69.0 ppm; C-5, 76.4 ppm; C-6, 62.4 ppm. Curdlan: C-1, 104.6 ppm; C-2, 73.9 ppm; C-3, 90.1 ppm; C-4, 70.2 ppm; C-5, 76.1 ppm; C-6, 62.5 ppm. Chitin: C-1, 104.0 ppm; C-2, 55.5 ppm; C-3, 73.5 ppm; C-4, 83.5 ppm; C-5, 76.0 ppm; C-6, 61.5 ppm; C-2 (methyl), 23.0 ppm; C-2 (carbonyl), 174.0 ppm.

effective against murine tumor.[7] We also reported on the effect of oral administration of the hot water extract of *G. frondosa*.[15] Based on the data presented here, it is suggested that the "native" form of the (1→3)-β-D-glucan is probably the active component of these preparations.

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Comparison of Binding Characteristics of Human and Bovine Serum Albumins with Benzoates over a Wide Range of Concentration

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The binding parameters of human serum albumin (HSA) and bovine serum albumin (BSA) with 14 benzoate derivatives and 2-naphthoate were determined by means of the ultracentrifugation method and the three-parameter equation previously proposed. The multiple regresssion analysis of the binding parameters revealed that the physico-chemical factors influencing the parameter α, which takes account of the increase in the binding capacity of albumin, were different in part between HSA and BSA. Moreover, the mean of the α values was greater with HSA than with BSA. This may indicate that HSA has a more flexible structure, and this may be consistent with lower contents of α-helix and β-sheet in the HSA molecule. Such differences were not recognized in the binding affinity of the serum albumins.

Keywords——serum albumin; binding equation; binding capacity; protein binding; ultracentrifugation method; substituted benzoate; multiple regression analysis

The interaction between serum albumin and ligands is often accompanied with conformational changes of the protein.[1] Previously, assuming that an increase in the degree of binding of serum albumin causes some changes in its higher-order structure which give rise to an increase in the capacity for binding, we[2] derived an equation with three parameters from Langmuir's equation. The validity of the equation was confirmed and the characteristics of the three binding parameters were examined by analysis of the data on bovine serum albumin (BSA) binding over a wide range of ligand concentration.

Since human serum albumin (HSA) was thought to be more flexible owing to its lower α-helix and β-sheet contents compared with BSA, greater conformational changes might be expected in the case of HSA. Therefore, in the present study, the data on the binding of 4-substituted benzoates and 2-naphthoate with HSA and BSA were analyzed by the use of the three-parameter equation, and the values and characteristics of the parameters, especially the one which takes account of the increase in the binding capacity, were compared.

Experimental

Materials——Human serum albumin (HSA, fraction V; Miles Laboratories Co., Elkhart, IN) and bovine serum albumin (BSA, fraction V; Armour Pharmaceutical Co., Kankakee, IL) were used. The molecular weight was assumed to be 67000 for both proteins, and the concentration was determined by measuring the absorbance at 280 nm using $E_{1\,cm}^{1\%}=5.30$[3] and 6.67[4] for HSA and BSA, respectively. 4-Ethoxybenzoate and 4-dimethylaminobenzoate were purchased from Tokyo Kasei Co., Tokyo. All other benzoates and 2-naphthoate were obtained from Wako Pure Chemical Industries, Osaka.

Ultracentrifugation (UC) Method——The general procedures were the same as those described previously.[5] The concentration of serum albumins was 2.08×10^{-5} M. All final solutions were prepared with 0.15 M Tris–HCl, pH 7.0. The concentrations of ligands and the wavelengths used for spectrophotometry are listed in Table I. All the

experiments were performed at 15 °C. Each point in the Scatchard plots represents the mean value of two experiments.

Calculation——Binding parameters were calculated on a PC-9801-E (NEC) personal computer. The characteristics of the three parameters in the binding equation were examined quantitatively by the use of multiple regression analysis. The predictor variables included the van der Waals volume (V_W),[6] hydrophilic effect (V_H),[6] and hydrophobic constant (π)[7] for the substituent, and pK_a.[8] Correlations and regression equations were calculated on a JEOL computer, model JEC-7E.

Results and Discussion

The data on binding of benzoates, 4-substituted benzoates, and 2-naphthoate to HSA and BSA measured by the ultracentrifugation method were plotted according to Scatchard.[9] As examples, the results for 4-NO_2- and 4-CH_3-benzoates are shown in Fig. 1. When the binding of ligands is measured over a wide range of ligand concentrations, in most cases the plot does not intersect with the abscissa at a constant point, but the number of sites appears to increase with increase of binding, as shown in Fig. 1. In the case of such binding data,

Table I. Concentration and Wavelength for Spectrophotometry of Benzoates and 2-Naphthoate

Ligand	Concn. (10^{-4} M)	λ (nm) for spectrophotometry
Benzoate	1.20—40.0	225
4-Cl-benzoate	0.10—50.0	235
4-F-benzoate	0.10—20.0	225
4-NO_2-benzoate	0.10—25.0	272
4-OH-benzoate	0.20—80.0	246
4-NH_2-benzoate	0.60—38.0	265
4-CN-benzoate	0.15—38.0	238
4-CH_3-benzoate	0.12—15.0	235
4-CH_3O-benzoate	0.10—25.0	248
4-CH_3CO-benzoate	0.15—30.0	254
4-C_2H_5-benzoate	0.10—20.0	235
4-C_2H_5O-benzoate	0.10—25.0	248
4-C_3H_7-benzoate	0.10—10.0	236
4-$(CH_3)_2N$-benzoate	0.10—25.0	290
2-Naphthoate	0.08—10.0	231

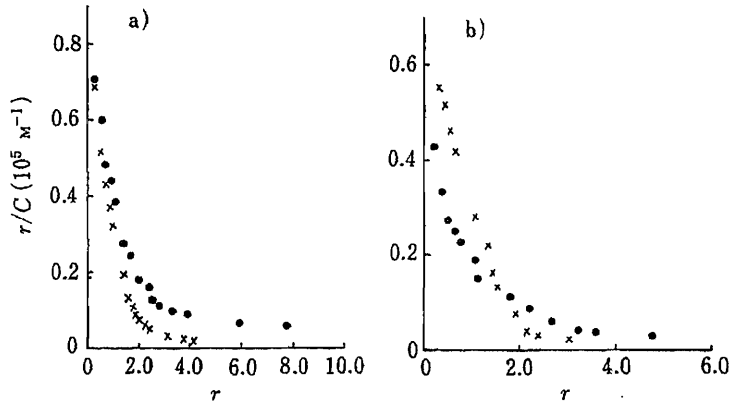

Fig. 1. Scatchard Plots for the Binding of 4-Nitrobenzoate and 4-Methylbenzoate to Serum Albumins

a) 4-Nitrobenzoate. b) 4-Methylbenzoate. ●, HSA; ×, BSA.

TABLE II. Parameters for Binding of Benzoate, 4-Substituted Derivatives of Benzoate, and 2-Naphthoate with HSA and BSA

Compound	HSA				BSA[a]				V_W[b]	V_H[b]	π[c]	pK_a[d]
	n_0	K ($10^4\,M^{-1}$)	α ($10^3\,M^{-1}$)	$\log K_{1:1}$	n_0	K ($10^4\,M^{-1}$)	α ($10^3\,M^{-1}$)	$\log K_{1:1}$				
Benzoate	1.39	1.49	1.01	4.36	1.48	1.41	1.03	4.35	0.785	0.0	0.0	4.21
4-Cl-benzoate	2.16	7.07	3.37	5.17	2.56	5.48	1.04	5.15	0.950	0.0	0.71	3.99
4-F-benzoate	1.56	3.11	2.67	4.72	3.19	1.18	1.43	4.58	0.831	0.0	0.14	4.14
4-NO_2-benzoate	1.92	3.99	4.72	4.91	2.01	2.93	1.13	4.78	0.994	0.31	−0.28	3.44
4-OH-benzoate	3.92	0.11	0.45	3.65	2.67	0.24	0.60	3.83	0.853	0.32	−0.67	4.58
4-NH_2-benzoate	3.41	0.04	0.49	3.19	2.16	0.07	0.57	3.25	0.884	0.59	−1.23	4.89
4-CN-benzoate	1.80	1.31	4.13	4.44	2.41	0.68	0.69	4.22	0.962	0.44	−0.57	3.55
4-CH_3-benzoate	2.09	1.67	2.03	4.56	1.80	3.73	0.87	4.83	0.939	0.0	0.56	4.37
4-CH_3O-benzoate	2.14	1.90	1.93	4.63	2.80	4.17	1.00	4.94	1.020	0.29	−0.02	4.49
4-CH_3CO-benzoate	2.98	0.80	2.03	4.39	2.85	1.27	1.40	4.57	1.114	0.56	−0.55	3.70
4-C_2H_5-benzoate	2.82	2.03	2.76	4.77	2.56	4.32	1.01	5.05	1.111	0.0	1.02	4.35
4-C_2H_5O-benzoate	2.27	3.17	2.07	4.87	1.94	7.05	2.31	5.14	1.173	0.29	0.38	4.80
4-C_3H_7-benzoate	2.17	6.27	7.40	5.15	2.08	9.26	5.31	5.30	1.256	0.0	1.55	4.34[e]
4-$(CH_3)_2N$-benzoate	2.30	1.06	1.70	4.41	2.52	1.09	1.09	4.45	1.208	0.45	0.18	6.03[f]
2-Naphthoate	2.51	14.64	3.82	5.57	2.18	21.10	4.05	5.63	1.205	0.0	1.32	4.16
Mean	2.36	3.24	2.71	4.59	2.35	4.27	1.57	4.67	1.019	0.22	0.17	4.34

a) The values were taken from Ref. 2 except those for 4-F, 4-C_2H_5, 4-C_2H_5O, and 4-C_3H_7 derivatives. *b*) Ref. 6. *c*) Ref. 7. *d*) Ref. 8 unless otherwise noted. *e*) Calculated from Hammett const. (σ_p). *f*) Ref. 11.

Karush's equation[10] generally used in protein binding studies is not applicable, and Eq. 1,[2] which we previously proposed for such binding data, was used for the analysis.

$$r=\{(n_0+\alpha C)KC\}/(1+KC) \quad (1)$$

where r is the average number of ligands per mole of protein, n_0 is the number of initial binding sites, α is a constant related to the increase in binding sites, K is the binding constant, and C is the unbound ligand concentration.

The binding parameters obtained are listed in Table II. The values for binding of 10 benzoates and 2-naphthoate with BSA were taken from our previous work.[2] Table II shows that the values of $\log K_{1:1}$ (affinity constant of protein–ligand 1 : 1 binding) for HSA and BSA are similar. On the other hand, the values of α for both albumins differ greatly. The means of the α values for 15 ligands were $2.71\times10^3\,\mathrm{M}^{-1}$ with HSA and $1.57\times10^3\,\mathrm{M}^{-1}$ with BSA. Since α is the constant which takes account of the increase in the binding capacity of albumin caused by the binding of ligands, it is suggested that HSA has a more flexible structure. This seems consistent with the lower contents of α-helix (BSA, 68%, HSA, 44—57.8%)[12-14] and β-sheet (BSA, 18%, HSA, 10%)[12,13] in HSA than in BSA.

Multiple regression analysis was applied to the above parameters to compare the binding characteristics of HSA with those of BSA. As the criterion variables, n_0, $\log\alpha$, and $\log K_{1:1}$ in Table II were used. The regression equations derived are shown in Table III. In these equations, the figures in parentheses are the 95% confidence limits, n is the number of data points, r is the correlation coefficient, and s is the standard error of the estimate.

As regards n_0, no equation where all the predictor variables were significant at $p<0.05$ was obtained for the binding of HSA or that of BSA.

As regards $\log\alpha$, good correlations were found with the combination of V_W and pK_a in the case of HSA (Eq. 2) and with the combination of V_W and V_H in the case of BSA (Eq. 5), as shown in Table III. The factors influencing $\log\alpha$ are thus different in part between HSA and BSA. Larger size of molecules increases the value of $\log\alpha$ for both albumins. However, less acidic (for HSA) or more hydrophilic (for BSA) character reduces the $\log\alpha$ value. This difference between HSA and BSA may be partly owing to the fact[15] that the net charge value at pH 7.4 is higher with HSA than with BSA.

TABLE III. Regression Equations for Characterization of the Binding Parameters

HSA:

$$\log\alpha=1.55(\pm0.87)^{b)}V_W-0.33(\pm0.21)^{b)}pK_a+3.20(\pm0.11)^{b)} \quad (2)$$
$$n=15,\quad r=0.801,^{b)}\quad s=0.218$$

$$\log K_{1:1}=2.51(\pm1.21)^{b)}V_W-1.53(\pm0.73)^{b)}V_H-0.34(\pm0.27)pK_a+3.84(\pm0.27)^{b)} \quad (3)$$
$$n=15,\quad r=0.909,^{b)}\quad s=0.277$$

$$\log K_{1:1}=0.64(\pm0.21)^{b)}\pi-0.31(\pm0.26)^{a)}pK_a+5.84(\pm1.16)^{b)} \quad (4)$$
$$n=15,\quad r=0.894,^{b)}\quad s=0.285$$

BSA:

$$\log\alpha=1.32(\pm0.64)^{b)}V_W-0.55(\pm0.43)^{a)}V_H+1.87(\pm0.66)^{b)} \quad (5)$$
$$n=15,\quad r=0.833,^{b)}\quad s=0.165$$

$$\log K_{1:1}=2.83(\pm0.99)^{b)}V_W-1.63(\pm0.64)^{b)}V_H-0.26(\pm0.24)^{a)}pK_a+3.26(\pm1.25)^{b)} \quad (6)$$
$$n=15,\quad r=0.934,^{b)}\quad s=0.244$$

$$\log K_{1:1}=0.69(\pm0.21)^{b)}\pi-0.22(\pm0.26)pK_a+5.50(\pm0.12)^{b)} \quad (7)$$
$$n=15,\quad r=0.906,^{b)}\quad s=0.276$$

a) Significant at $p<0.05$. b) Highly significant at $p<0.01$.

Regarding $\log K_{1:1}$, several significant equations, Eqs. 3 and 4 for HSA and Eqs. 6 and 7 for BSA in Table III, were derived. These equations clearly indicate that bulky and hydrophobic molecules with strong acidity are bound favorably to both albumins.

In conclusion, the three-parameter equation which we previously proposed for the description of ligand binding behavior over a wide range of ligand concentration fitted well to the binding data of HSA with benzoates and 2-naphthoate. The binding parameter α taking account of the increase in the binding capacity of albumin was related to the size of ligand molecule and its acidity (for HSA) or hydrophobicity (for BSA). The greater values of α observed with HSA binding may indicate that HSA is more flexible than BSA.

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Communications to the Editor

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AN EFFICIENT SYNTHESIS OF WF-3681, A NOVEL ALDOSE REDUCTASE INHIBITOR, AND ITS RELATED COMPOUNDS

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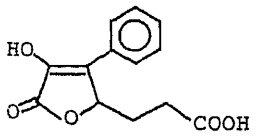
WF-3681 (1a), an aldose reductase inhibitor, and its related compounds (1b-1j) have been synthesized by aldol condensation of phenylpyruvates and ω-formylalkanoates as a key step.

KEYWORDS —— fungal metabolite; aldose reductase inhibitor; aldol condensation; phenylpyruvate; ω-formylalkanoate

We previously described the structure and synthesis of WF-3681 (1a), a novel aldose reductase inhibitor isolated from *Chaetomella* species.[2,3] Here we report an expeditious synthesis of this inhibitor and its related compounds and analyze their biological activity.



1a

We anticipated that the α-hydroxybutenolide ring system of WF-3681 could be constructed by aldol condensation of phenylpyruvate (e.g., 2a) with 3-formylpropionate (e.g., 3a) followed by lactonization of the resulting hydroxy keto ester 4 as depicted in Chart 1.[4] Hydrolysis of the side-chain ester group in the product 5a would afford compound 1a, which was expected to be identical in *all respects* with the natural WF-3681, since the latter had been isolated as *a racemic mixture*.[3]

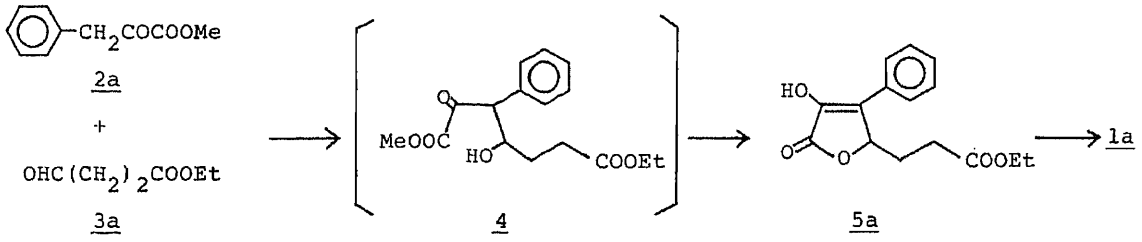
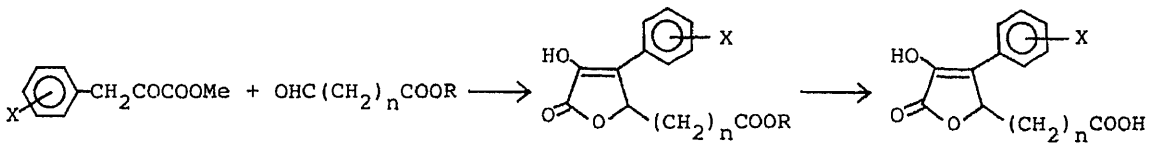


Chart 1

Methyl phenylpyruvate (2a) (mp 55-60°C) was prepared by methylating phenylpyruvic acid (MeI/DBU/DMF, 0°C, 87%).[5] Ethyl 3-formylpropionate (3a) [bp 68-78°C (7mmHg)] was prepared according to the method reported in the literature.[6] The key aldol condensation was conducted by stirring 2a and 3a in the presence of DBU in DMF at 0°C for 2.5 h. Under these conditions, the desired product 5a (mp 116-118°C) was obtained directly in 72% yield. The structure was characterized on the basis of its physical data [EIMS m/z 276 (M^+); IR(nujol) 3270,



2b X=4-Cl
c X=4-Me
d X=4-OCH_2Ph
e X=3,4-diCl
f X=3-CF_3,4-OMe

3b R=Bu^n; n=0
c R=Me; n=3

5b X=4-Cl; R=Et; n=2
c X=4-Me; R=Et; n=2
d X=4-OCH_2Ph; R=Et; n=2
e X=3,4-diCl; R=Et; n=2
f X=3-CF_3,4-OMe; R=Et; n=2
g X=H; R=Bu^n; n=0
h X=H; R=Me; n=3

1b X=4-Cl; n=2
c X=4-Me; n=2
d X=4-OCH_2Ph; n=2
e X=3,4-diCl; n=2
f X=3-CF_3,4-OMe; n=2
g X=H; n=0
h X=H; n=3
i X=4-OH; n=2

Chart 2

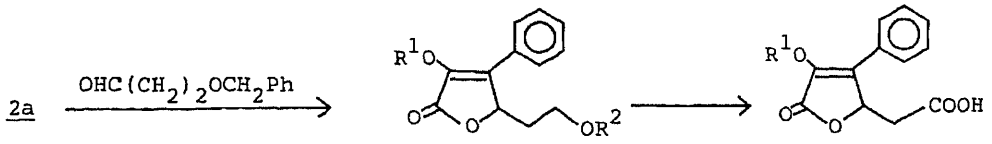
Table I. Synthesis of Compounds Related to WF-3681

Starting material	Aldol condensation			Hydrolysis		
	Product	mp(°C)	Yield(%)	Product	mp(°C)	Yield(%)
2b[a] + 3a	5b	121-122	60	1b	181-182	96
2c[a] + 3a	5c	108-109	61	1c	168-169	100
2d[a] + 3a	5d	130-131	72	1d	198-199	60
2e[a] + 3a	5e	109-111	70	1e	179-180	86
2f[b] + 3a	5f	168-169	51	1f	224-226	87
2a + 3b[c]	5g	108-109	87	1g[d]	189-190	45
2a + 3c[c]	5h	78-79	45	1h	179-180	88
				1i[e]	251-253	57

a) Prepared from methyl 2,2-dimethoxy-3-(substituted phenyl)propionates, synthesized according to the known procedure,[7] by heating in HCO_2H (65-70°C).
b) Prepared in the same way as for 2a.
c) Prepared according to the known procedures.[8]
d) Hydrolysis was achieved by using 1N NaOH/THF at room temp.
e) Prepared by treating 5d with 3N HCl/AcOH(100°C,4h).

1740, 1705 cm^{-1}; NMR($CDCl_3$) δ: 1.27 (3H, t, J=7Hz), 1.75 (1H, m), 2.32-2.77 (3H, m), 4.16 (2H, q, J=7Hz), 5.51 (1H, dd, J=2, 9Hz), 6.74 (1H, s)]. Acid hydrolysis of 5a (3N HCl/AcOH, 100°C, 1h) yielded WF-3681 (1a)(100%), which was identified with the natural product[3] in all respects.

The synthesis of 1a is highly efficient and provides the amounts necessary for detailed biological tests. Moreover, this method is applicable to the preparation of compounds related to 1a. Some compounds having substituents on the benzene ring (1b-1f and 1i) and modified carboxylic acid side-chains (1g and 1h) were thus prepared (Chart 2) and are listed in Table I. However, we were unable to achieve the aldol reaction using ethyl formylacetate,[9] probably due to the formation of an anion on the formylacetate rather than the phenylpyruvate. Therefore, we chose, for the preparation of 1j, 3-benzyloxypropionaldehyde[10] as the starting material and carried out the reaction with methyl phenylpyruvate under the above conditions to obtain α-hydroxybutenolide 6 (mp 112-113°C, 69%). Conversion of 6 to 1j (mp 204-205°C) was achieved via 7 (oil), 8 (mp 82-84°C), and 9 (mp 159-160°C) by a sequence of reactions (1. CH_2N_2/MeOH, 100%; 2. Pd-black/HCO_2H-MeOH, 90%; 3. CrO_3/H_2SO_4/acetone, 65%; 4. BBr_3/CH_2Cl_2, 26%) (Chart 3).



6 R^1=H, R^2=CH_2Ph
7 R^1=Me, R^2=CH_2Ph
8 R^1=Me, R^2=H

9 R^1=Me
1j R^1=H

Chart 3

Table II. Inhibition of Rabbit Lens Aldose Reductase[a]

Compound	IC_{50} (M)	Compound	IC_{50} (M)
1a	2.5×10^{-7}	1f	5.1×10^{-8}
1b	9.2×10^{-8}	1i	1.6×10^{-7}
1c	8.4×10^{-8}	1g	$>1.0 \times 10^{-5}$ [b]
1d	4.9×10^{-8}	1j	$>1.0 \times 10^{-5}$ [c]
1e	9.8×10^{-8}	1h	1.0×10^{-5}

a) Enzyme activity was assayed by a modified method[2] described in the literature.[11]
b) A 45% inhibition at 1.0×10^{-5}M.
c) A 44% inhibition at 1.0×10^{-5}M.

The aldose reductase inhibitory activity of the new compounds above are shown in comparison with that of 1a in Table II. All the substituted benzene derivatives were more active than WF-3681, showing that the introduction of the lipophilicity tends to increase the activity. Modification of the carboxylic acid side-chain was found to decrease the activity, indicating that the side-chain length plays an important part in the activity.

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TOTAL SYNTHESIS OF ESTERS OF AK-TOXIN II AND AF-TOXIN IIc STARTING FROM VITAMIN C AS A CHIRAL MATERIAL

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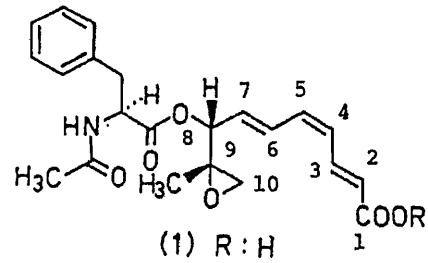
Total synthesis of the methyl esters of AK-toxin II and AF-toxin IIc, host-specific toxins to the susceptible cultivars Japanese pear and strawberry, was accomplished in optically active forms using vitamin C as a chiral material.

KEYWORDS —— phytotoxicity; host-specific toxin; vitamin C; chiral synthon; isoleucine; Wadsworth-Emmons reaction; Mitsunobu reaction

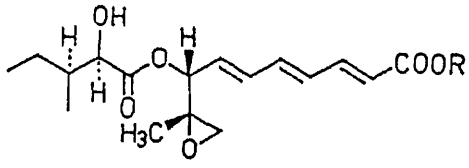
Previously, we reported the synthesis of the congeners of AK-toxin II[1] and AF-toxin IIc[2] methyl esters and revealed two chiral centers of the trienoic acid moiety of AF-toxin IIc to be at least of the C_8(R) and C_9(S) configuration.[3] However, the stereochemistry of the 2-hydroxy-3-methylpentanoic acid moiety of the toxin remained to be elucidated.[4] In continuing the synthesis of the toxins, AK- and AF-toxins produced by *Alternaria kikuchiana* and *A. alutanata*, respectively, we report total synthesis of the methyl esters (2) and (4) of AK-toxin II (1) and AF-toxin IIc (3) in optically active forms from vitamin C as a chiral starting material.

As indicated previously,[3] vitamin C is easily converted to the aldehyde (5) The known all-*trans*-trienoic ester (7)[3] was obtained, though as a minor product, in one step by a Wadsworth-Emmons reaction of (5) with methyl 4-dimethylphosphonocrotonate. Unexpectedly, the major product of the above reaction was the cyclic compound (6) produced by participation of two double bonds in the reaction intermediate and by a hydrogen rearrangement as depicted in Chart 1. On the other hand, treatment of (5) with methyl 4-triphenylphosphoniumcrotonate gave the *cis*- (8) and the *trans*-trienoic acid ester (7) in 85% yield in 3:1 ratio. Oxidation of (8) with *m*-chloroperbenzoic acid gave two diastereoisomeric oxides (9) and (10) in 38% and 43% yield, respectively. The structures of these oxides were determined by comparing their ^{1}H-NMR spectral data with those of natural AK-toxins and their congeners.[3]

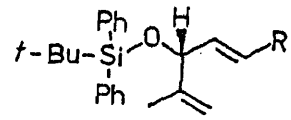
De-protection of the silyl group in the oxide (9) with tetrabutylammonium fluoride gave the alcohol (11) in good yield. The alcohol was esterified with N-acetyl-L-phenylalanine and dicyclohexylcarbodiimide in the presence of 4-pyrrolidinopyridine[5] to give a mixture of AK-toxin II methyl ester (2)[6] and its epimer



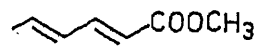
(1) R : H
(2) R : CH_3

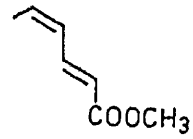


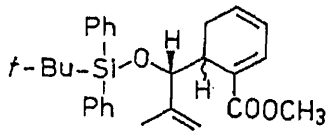
(3) R : H
(4) R : CH_3



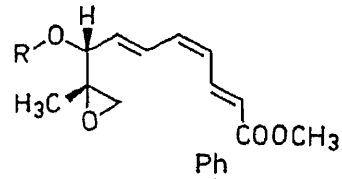
(5) R : CHO

(7) R: 

(8) R: 

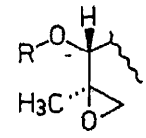


(6)

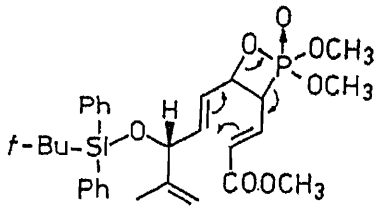


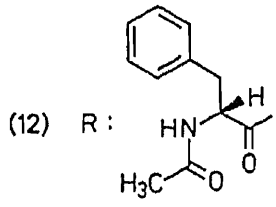
(9) R : t-Bu-Si(Ph)-Ph

(11) R : H



(10) R : t-Bu-Si(Ph)-Ph





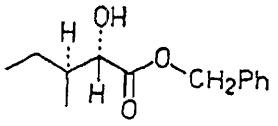
(12) R :

Chart 1

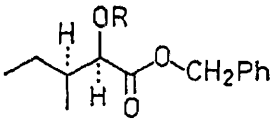
(12). This was a result of an inevitable racemization caused by activation of the carboxylic acid group of N-acetylphenylalanine. The stereochemistry of the α-carbon of phenylalanine in the esters (2) and (12) was confirmed by the L- and D-amino acid determination method.[7] The synthetic AK-toxin II methyl ester (2) was identical with the natural one in all respects, confirming the total synthesis of the toxin methyl ester from vitamin C as a chiral starting material.[8]

Next, we focused our attention on the total synthesis of AF-toxin IIc methyl ester (4). Treatment of the hydroxy-ester (13),[3] $[\alpha]_D^{27}$ -11.8° (c=1.0, EtOH),

with formic acid and diethyl azodicarboxylate (Mitsunobu reaction) gave the formyl ester (14), $[\alpha]_D^{23}$ +23.3° (c=1.2, $CHCl_3$), which, under mild alkaline hydrolysis, gave the hydroxy-ester (15), $[\alpha]_D^{23}$ +9° (c=1.1, EtOH) in 80% yield. Protection of a hydroxyl group as *tert*-butyldiphenylsilyl ether followed by hydrogenation gave the acid (16), $[\alpha]_D^{22}$ +18.5° (c=1.0, EtOH). The all-*trans*-ester (17)[3] was subjected to acylation with the acid (16) to furnish the ester (18) in 75% yield without racemization by Hassner and Alexanian's method.[5] Treatment of (18) with tetrabutylammonium fluoride in tetrahydrofuran gave AF-toxin IIc methyl ester in80% yield. The ^{1}H-NMR spectrum[9] (400MHz in $CDCl_3$) showed signals identical with those of the reported values,[2] thus confirming the synthesis of AF-toxin IIc methyl ester.[10]

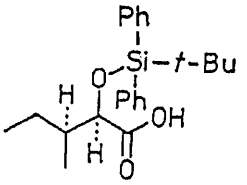


(13)

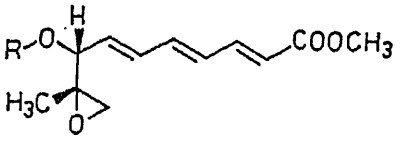


(14) R : CHO

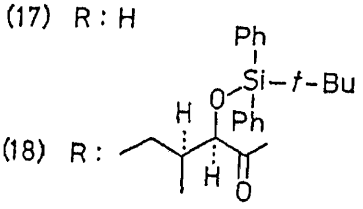
(15) R : H



(16)



(17) R : H



(18) R :

Chart 2

ACKNOWLEDGEMENT This study was supported in part by a Grant in Aid for Scientific Research (No. 61571007) from the Ministry of Education, Science and Culture of Japan.

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2) S. Nakatsuka, K. Ueda, T. Goto, M. Yamamoto, S. Nishimura, and K. Kohmoto, *Tetrahedron Letters*, 27, 2753 (1986).

3) H. Irie, K. Matsumoto, and Y. Zhang, *Chem. Pharm. Bull.*, 34, 2668 (1986).

4) When we synthesized the compound, which had an entire carbon skeleton and functionalities corresponding to AF-toxin IIc and was toxic to the leaves of Nijisseiki (japanese pear), we thought the synthesis of the toxin had been achieved. Then we requested the spectroscopic data from the Nagoya group but did not receive the data. After our paper[3] indicating the stereostructure of AF-toxin IIc was accepted, the Nagoya group reported the ^{1}H-NMR data of AF-toxin IIc methyl ester. Then we realized our compound was not identical with the natural toxin, since the chemical shifts of the primary and secondary methyl groups of our compound were quite different from those of genuine toxin. Because of its toxicity, the synthetic compound was thought to have the right stereostructure in the part of the trienoic acid moiety but the wrong configuration in the 2-hydroxy-3-methylpentanoic acid part.

5) A. Hassner and V. Alexanian, *Tetrahedron Letters*, 1978, 4475.

6) ^{1}H-NMR (δ) ($CDCl_3$) 1.31 (3H, s), 2.01 (3H, s), 2.61 (1H, d, J=4.6Hz), 2.75 (1H, d, J=4.6Hz), 3.12 (2H, d, J=6.2Hz), 3.77 (3H, s), 5.27 (1H, d, J=7.7Hz), 5.70 (1H, dd, J=15.0 and 7.7Hz), 5.92 (1H, broaded and overlapped on δ 5.95 signal, *NH*), 5.95 (1H, d, J=15.4Hz), 6.20 (1H, t, J=11.5Hz), 6.29 (1H, t, J=11.5Hz), 6.84 (1H, dd, J=15.0 and 11.5Hz), 7.08 (2H, dd, J=7.9 and 1.6Hz), 7.35-7.20 (3H, m), 7.70 (1H, dd, J=15.4 and 11.5Hz).

7) C.R. Shoaf, K.J. Isselbacher, and W.D. Heizer, *Anal. Biochem.*, 61, 72 (1974).

8) To our knowledge, there have been only two reports concerning the synthesis of natural products using vitamin C as a chiral starting material. M.E. Jung and T.J. Shaw, *J. Am. Chem. Soc.*, 102, 6304 (1980); C.C. Wei, S.D. Bernardo, J.P. Tengi, J. Borgese, and M. Weigele, *J. Org. Chem.*, 50, 3462 (1985).

9) The ^{1}H-NMR data of synthetic AF-toxin IIc methyl ester; (the reported data are indicated in parenthesis) (δ) ($CDCl_3$), 0.85 (3H, d, J=7.0Hz), (0.87); 0.98 (3H, t, J=7.4Hz), (0.97); 1.36 (2H, m) (1.36); 1.36 (3H, s), (1.36); 1.84 (1H, m), (1.84); 2.61 (1H, d, J=5.9Hz, OH), (2.60); 2.63 (1H, d, J=4.8Hz), (2.62); 2.79 (1H, d, J=4.8Hz), (2.78); 3.76 (3H, s), (3.75); 4.23 (1H, dd, J=2.9 and 5.9Hz), (4.21); 5.35 (1H, d, J=7.3Hz), (5.34); 5.82 (1H, dd, J=7.3 and 14.8Hz), (5.82); 5.94 (1H, d, J=15.4Hz), (5.94); 6.38 (1H, dd, J=11.0 and 14.5Hz), (6.38); 6.43 (1H, dd, J =10.8 and 14.8Hz), (6.43); 6.53 (1H, dd, J=10.8 and 14.5Hz), (6.53); 7.29 (1H, dd, J=11.0 and 14.4Hz), (7.29).

10) The ^{1}H-NMR data of AF-toxin IIc methyl ester were not available, when our paper (ref. 3) was in press.

(Received March 28, 1987)

Communications to the Editor

[Chem. Pharm. Bull. 35(6)2602—2604(1987)]

SYNTHESIS OF COCCULOLIDINE SKELETON, A NON-AROMATIC NOR-ERYTHRINAN ALKALOID[1)]

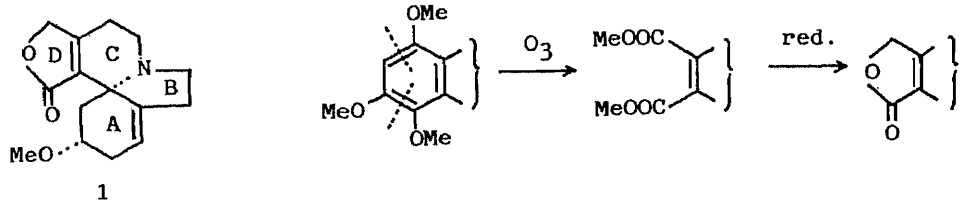
Kimiaki Isobe,*,[a] Kunihiko Mohri,[a] Manabu Maeda,[a] Taroh Takeda[a] and Yoshisuke Tsuda[b]

Showa College of Pharmaceutical Sciences,[a] 5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan and Faculty of Pharmaceutical Sciences,[b] Kanazawa University, 13-1 Takara-machi, Kanazawa 920, Japan

The skeleton of cocculolidine, a non-aromatic nor-erythrinan alkaloid, was synthesized in four steps from 14,15,17-trimethoxy-8-oxo-erythrinan by utilizing ozonolytic cleavage of an aromatic ring as a crucial step.

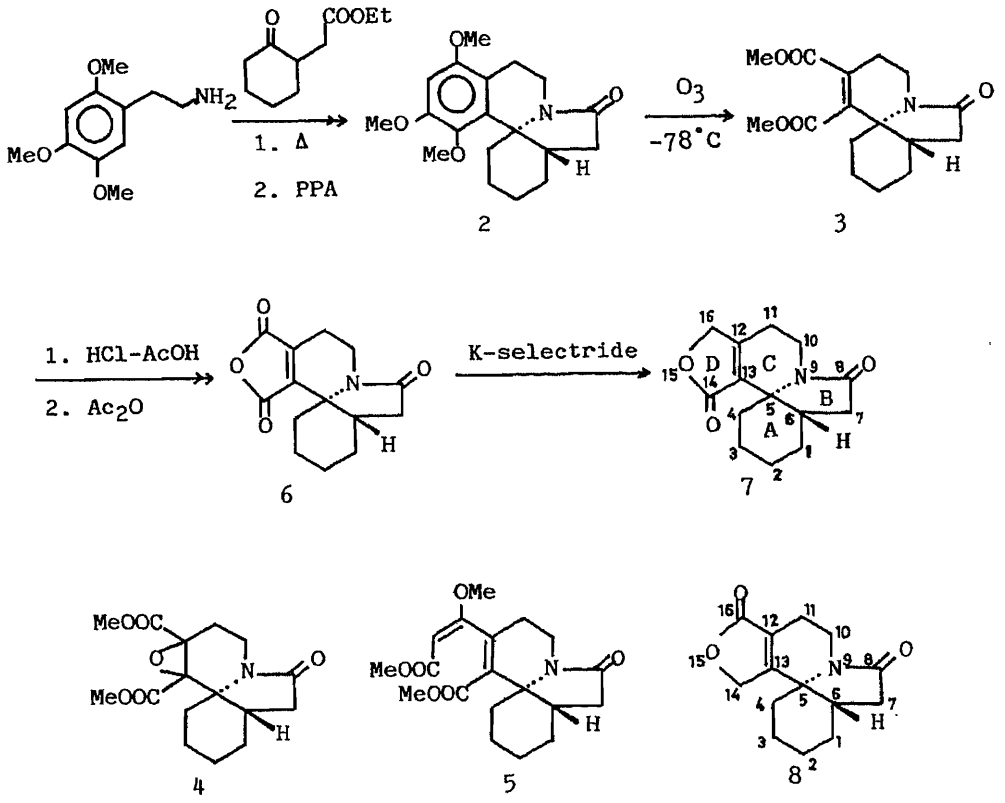
KEYWORDS —— cocculolidine; non-aromatic erythrinan alkaloid; nor-erythrinan alkaloid; ozonolysis; aromatic ring cleavage; regioselective reduction

Cocculolidine (1) is the insecticidal alkaloid isolated from fresh leaves of *Cocculus trilobus* DC. (Menispermaceae).[2)] Structurally it belongs to the group of non-aromatic nor-erythrinan alkaloids and characteristically has a γ-lactone instead of an aromatic ring at ring D. No report has appeared for the synthesis of the alkaloid or its skeleton. In this communication we describe the synthesis of its skeleton from an aromatic erythrinan by utilizing the ozonolytic cleavage of the aromatic ring as a crucial step.



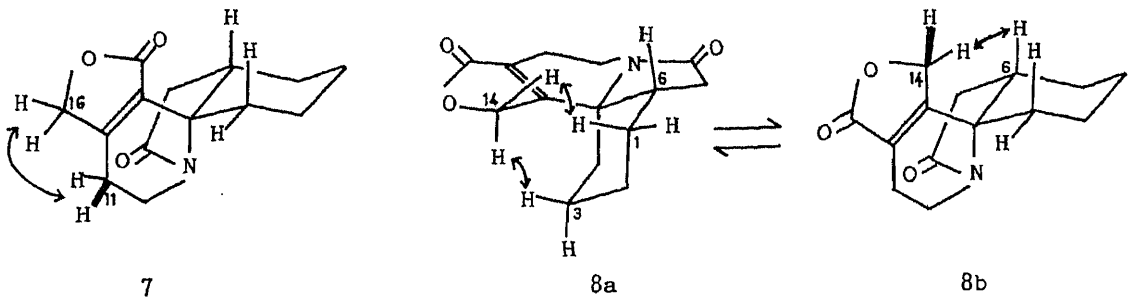
1

14,15,17-Trimethoxy-8-oxo-*cis*-erythrinan (2)[3)] was prepared in 51% yield by condensation of 2,4,5-trimethoxyphenylethylamine and ethyl cyclohexanone-2-acetate in toluene (reflux, 8 h) followed by cyclization with an excess of PPA (100°C, 6 h).[4)] Ozonolysis of 2 in CH_2Cl_2 at -78°C for 90 min gave the bisnor-diester (3)[3)] and the epoxide (4)[3)] in 63 and 12% yield, while the ozonolysis in the presence of boron trifluoride etherate as a regulator[5)] gave 3 and the seco-ester (5)[3)] in 48 and 22% yield, respectively. The structures of these products were elucidated from their spectral data.



The diester (3) was hydrolyzed with 70% AcOH containing 10% HCl (100°C, 18 h) to the di-acid which on heating with acetic anhydride was converted to the anhydride (6) (IR: 1850, 1775, 1688 cm^{-1}) almost quantitatively.

Reduction of 6 with zinc borohydride in tetrahydrofuran (THF) at room temperature afforded a γ-lactone (7)[3] (31%) and an isomeric lactone (8)[3] (11%). On the other hand, reduction of 6 with potassium tri-*sec*-butylborohydride (K-selectride) in THF at room temperature gave 7 in 70% yield as the sole product. A similar regioselective reduction of an anhydride at the less hindered carbonyl group with a sufficiently bulky hydride has a precedent.[6]



The structure of the γ-lactones 7 and 8 were firmly established on the basis of their NOE difference spectra. Firstly, all proton signals of 7 and 8 were assigned from their COSY spectra. Irradiation at δ 4.66 (s, -COOC$\underline{H}_2$C=) in 7 resulted in a clear NOE enhancement on the C_{11}-protons (δ 2.52 and 2.28). On the other hand, irradiation of the C_{14}-protons at δ 4.98 (dt) and 4.91 (ddd) in 8 produced no effect on the C_{11}-protons (δ ca. 2.41 and 2.32), but resulted instead in an enhancement on the axial protons (δ 1.59 and 1.20) at C_1 and C_3 as well as the proton at C_6 (ca. δ 2.37). This suggests that 8 exists as an equilibration of the conformations **8a** and **8b**. Thus it was concluded that the lactone (7) has the the cocculolidine skeleton.

The above synthesis provides the first synthesis of the non-aromatic nor-erythrinan skeleton.

ACKNOWLEGDGEMENT The authors thank Prof. T. Kikuchi and Dr. S. Kadota, Toyama Medical and Pharmaceutical University, for the measurement of the COSY and NOE difference spectra.

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3) All new compounds in this communication had satisfactory spectral data. IR were taken in KBr or $CHCl_3$ and given in cm^{-1}, ^{1}H-NMR were at 400 and/or 100 MHz and given in δ, and High-resolution MS were measured at 30 eV.

2. mp 120-124°C; $C_{19}H_{25}NO_4$; IR: 1690; ^{1}H-NMR: 6.42 (1H, s, Ar-H), 3.89, 3.87, 3.77 (each 3H, s, OCH_3).

3. Oil; $C_{16}H_{21}NO_5$; IR: 1730, 1698, 1648; ^{1}H-NMR: 3.82, 3.73 (each 3H, s, CH_3).

4. mp 119-121°C; $C_{16}H_{21}NO_6$; IR: 1750, 1740, 1690; ^{1}H-NMR: 3.80, 3.77 (each 3H, s, OCH_3).

5. Oil; $C_{19}H_{25}NO_6$; IR: 1775, 1720, 1680, 1600; ^{1}H-NMR: 5.00 (1H, s, =CH), 3.66, 3.65, 3.64 (each 3H, s, OCH_3).

7. Oil; $C_{14}H_{17}NO_3$; IR: 1759, 1680; UV λ_{max}^{MeOH}nm (ε): 213 (9800); ^{1}H-NMR: 4.66 (2H, s), -COO-CH_2-, 4.25 (1H, dd, J=13.5, 7.5 Hz) and 2.93 (1H, ddd, 13.5, 11, 5 Hz), C_{10}-H_2, 2.52 (1H, ddd, J=18.5, 11, 7.5 Hz) and 2.28 (1H, dd J=18.5, 5 Hz), C_{11}-H_2.

8. mp 207-209°C; $C_{14}H_{17}NO_3$; IR: 1750, 1690; UV λ_{max}^{MeOH}nm (ε): 206 (15500); ^{1}H-NMR: 4.98 (1H, dt, J=17.1, 2.8 Hz) and 4.91 (1H, ddd, J=17.1, 3.4, 1.7 Hz), -COO-CH_2-, 4.36 (1H, dd, J=13.5, 6.5 Hz) and 2.88 (1H, m), C_{10}-H_2, 2.41 (1H, m) and 2.32 (1H, m), C_{11}-H_2.

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Communications to the Editor

[Chem. Pharm. Bull. 35(6)2605—2608(1987)]

REACTION OF ORGANOMETALLIC REAGENTS WITH 2'- AND 3'-KETOURIDINE DERIVATIVES: SYNTHESIS OF URACIL NUCLEOSIDES BRANCHED AT THE 2'- AND 3'-POSITIONS

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The reaction of organolithium, Grignard, and organoaluminum reagents with 2'- and 3'-ketouridine derivatives was examined. For the reaction of 2',5'-bis-*O*-(tert-butyldimethylsilyl)-3'-ketouridine, both organolithiums and organoaluminums seem to be practically useful. But only organoaluminums gave satisfactory yields in the reaction of 3',5'-*O*-(tetraisopropyldisiloxan-1,3-diyl)-2'-ketouridine.

KEYWORDS —— organoaluminum reagent; organolithium reagent; Grignard reagent; ketouridine; branched-chain sugar nucleoside; arabinofuranosyluracil; xylofuranosyluracil

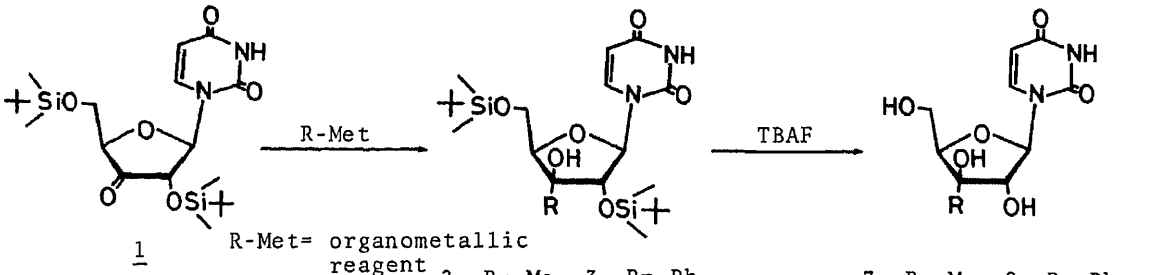
Since the first report[1] on the synthesis of 2'- and 3'-*C*-methyladenosines, nucleosides bearing branched-chain sugars have been known to be an important family, not only as biologically active compounds[2,3] but also for elucidation of enzyme recognition of substrates.[4-6] Most compounds in this category have been synthesized by the classical condensation method.[1-5] However, the usefulness of this approach is limited by the number of steps required to prepare the requisite branched-chain sugars. Less frequently these compounds have been prepared from easily accessible naturally-occurring nucleosides.[7]

One may easily anticipate that the reaction of organometallic reagents with ketonucleosides would constitute a simple and straightforward entry to these compounds. However, Cook and Moffatt reported that an attempt to treat 2',5'-di-*O*-trityl-3'-ketouridine with either methyl Grignards or methyllithium under various conditions did not give any alkylation product. Only borohydride reduction was successful.[8] Since then, no investigation has dealt with such an approach to branched-chain sugar nucleosides, to the best of our knowledge.[9]

As part of our continuing work on the use of organometallics for the modification of nucleosides,[10] we thought that the reported reactions of 3'-ketouridine should be reinvestigated by changing the protecting group in the sugar moiety, since organometallics such as Grignard reagents and alkyllithiums can be regarded as Lewis acids.[11]

With the success of the tert-butyldimethylsilyl (TBDMS) protecting group in our lithiation studies, 2',5'-bis-*O*-TBDMS-3'-ketouridine (1)[12] was selected as a starting material in the present study.

When 1 was treated with 3 eq of MeLi in THF for 3 h at below −70°C, the expected alkylation product (2) was obtained in 79% yield after quenching with AcOH followed by chromatographic purification through a Florisil® column (benzene:EtOAc = 10:1). Though similar treatment of 1 with PhLi gave a comparable yield (Table I, entry 2), the use of BuLi significantly decreased the yield (entry 3), giving several decomposition products.[13] This could be rationalized in terms of a higher basicity of BuLi which would alter the reaction course in favour of elimination.



1

R-Met= organometallic reagent

2 R= Me, 3 R= Ph,
4 R= Bu, 5 R= Et,
6 R= C≡CPh

7 R= Me, 8 R= Ph,
9 R= Bu, 10 R= Et,
11 R= C≡CPh

Chart 1

Table I. Synthesis of 3'-C-Substituted 2',5'-bis-O-TBDMS Xylofuranosyluracils

Entry	Organometallic reagent	R	Product	Yield(%)
1	MeLi	Me	2	79
2	PhLi	Ph	3	72
3	BuLi	Bu	4	33
4	MeMgBr	Me	2	37
5	EtMgBr	Et	5	24
6	PhMgBr	Ph	3	30
7	Me_3Al	Me	2	64
8	Ph_3Al	Ph	8	26*
9	$PhC{\equiv}CAlEt_2$	C≡CPh	6	58

* Overall yield of 8 from 1.

On the other hand, when Grignard reagents (3 eq) were used in the above reaction, hardly any decomposed products were formed. But as can be seen from Table I (entries 4, 5, and 6), the yields of products (2, 3, and 5) were uniformly low.[14]

The reaction of 1 with organoaluminum reagents was examined next using CH_2Cl_2 as a solvent. Though 1 was not susceptible to the reaction of Me_3Al (3 eq) at below −70°C, the expected carbalumination was achieved by conducting the reaction at room temperature, producing 2 in good yield (entry 7). Ph_3Al and $PhC{\equiv}CAlEt_2$ also worked to effect carbalumination of 1 (entries 8 and 9). It should be noted that even when these reactions were conducted at refluxing temperature, essentially no decomposition took place, showing the highly oxygenophilic character of organoaluminum reagents.

The products 2-6 obtained in the above reaction were deprotected in THF with

tetrabutylammonium fluoride (TBAF) to give the corresponding free 3'-C-substituted xylofuranosyluracils (7-11) in high yields.

Application of this approach to the synthesis of 2'-C-substituted arabinofuranosyluracils was then investigated using the organometallics listed in Table I and 3',5'-O-(tetraisopropyldisiloxan-1,3-diyl)-2'-ketouridine (12).[15]

As was expected from the reported instability of 2'-ketouridine,[8] both the organolithium and the Grignard reagents were practically inefficient, giving a complex mixture of products. Especially in the reactions of organolithium reagents, instantaneous precipitation occurred upon addition of the reagent to a THF solution of 12 (below −70°C).

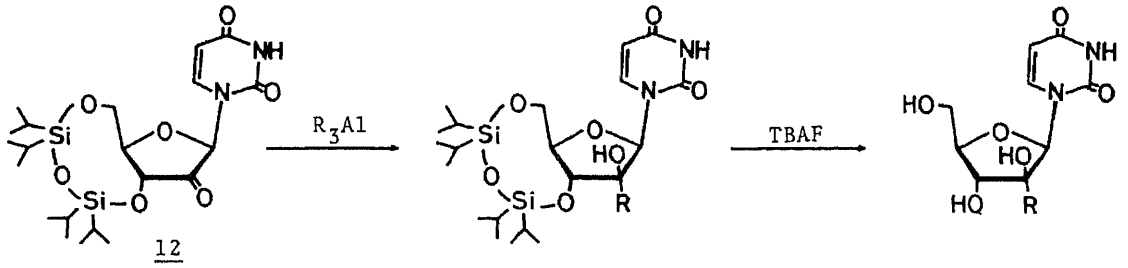


Chart 2

In contrast, the use of organoaluminum reagents again gave satisfactory results. Thus, when 12 was treated with 3 eq of Me_3Al in CH_2Cl_2 at room temperature for 1 h, the corresponding 2'-C-methylated product (13) was obtained in 98% yield. A similar reaction with $PhC{\equiv}CAlEt_2$ gave a 79% yield of 14. With Ph_3Al, the initially formed 15 could not be isolated in pure form, but deprotection with TBAF gave 2'-C-phenyl arabinofuranosyluracil (16) in 30% overall yield from 12. Compounds 13 and 14 were also treated with TBAF to furnish 17 and 18, respectively, in high yields.

Finally, the stereochemical reaction course of all of the organometallics involved in the present study was assumed to be alpha in corroboration with the X-ray crystallographic analysis of 16,[16] the result of which is depicted in Fig. 1.

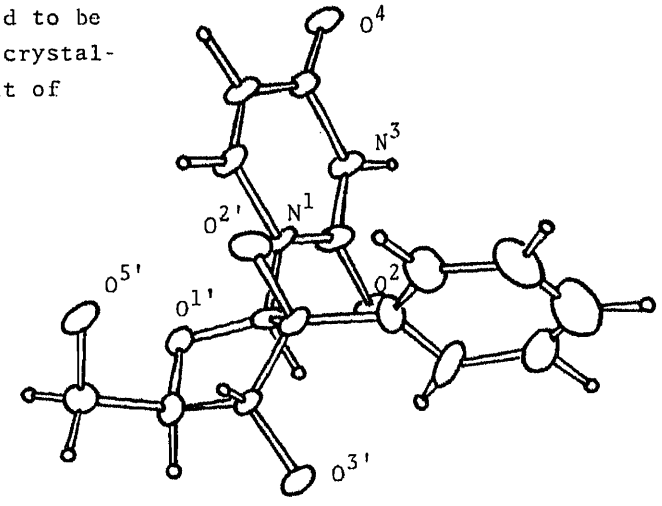


Fig. 1. Molecular Structure of 2'-C-Phenyl Arabinofuranosyluracil (16)[17]

ACKNOWLEDGEMENT

The authors thank Professor J. M. J. Tronchet, Université de Genève, for his cooperation through a private communication concerning the current status of the chemistry of branched-chain sugar nucleosides.

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17) Hydrogens are located on the calculated positions.

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Communications to the Editor

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ENANTIOSELECTIVE REDUCTION OF FLUOROALKYL ALKYNYL KETONES: ENORMOUS ELECTRONIC EFFECT OF THE TRIFLUOROMETHYL GROUP

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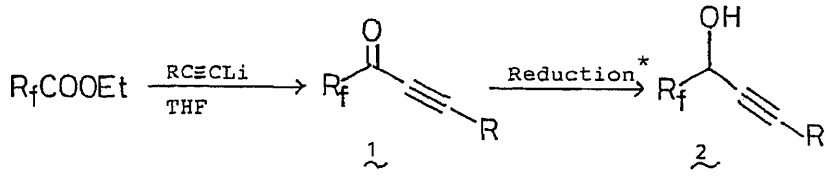
Fluoroalkyl alkynyl ketones (1a-c) were reduced enantioselectively by (R)-BINAL-H and (S)-Alpine-Borane to give optically active alcohols (2a-c). In the BINAL-H reduction, the trifluoromethyl group exerted an electronic effect on the enantioselectivity of the reagent.

KEYWORDS ——— asymmetric reduction; fluoroalkyl alkynyl ketone; electronic effect; transition state; fluoroalkyl group

Recent advances in the synthetic methodologies of enantioselective reactions have opened many possibilities for synthesizing highly functionalized molecules in optically pure form.[1] One remarkable advancement in enantioselective reactions is the reduction of a prochiral ketone to a chiral alcohol with a chiral reagent,[2] or by the microbial method.[3] Recently, microbial reduction has been carried out on fluoroalkyl ketone derivatives and the results indicate that the microbial method is the promising way to create optically active fluoroalkyl alkyl carbinols.[4] The present paper reports the enantioselective reduction of a series of fluoroalkyl alkynyl ketones (1a-c) with chiral reagents and the effects of the fluoroalkyl groups (trifluoro-, difluoro- and monofluoromethyl) on enantioselectivity. Optically active fluoroalkyl alkynyl carbinols (2a-c) are considered important building blocks for the synthesis of polyfunctionalized fluorine-containing molecules such as fluorinated sugar derivatives. Furthermore, it is worthwhile to clarify the effects of fluoroalkyl groups on asymmetric reduction. B-3-pinanyl-9-borabicyclo[3.3.1]-nonane (Alpine-Borane)[5] and binaphthol-modified aluminum hydride reagent (BINAL-H)[6] were used as reducing reagents since the transition states of both reductions have been well characterized. The selectivity of the Alpine-Borane reagent has been noted to be entirely based on a steric effect and that of BINAL-H based on an electronic effect. Electronegative and small fluorine atom(s) may have some effects different from the hydrocarbon compounds in enantioselective reductions. The starting ketones (1a-c) were prepared by reactions of the corresponding esters with lithium acetylide (-78°C～-23°C) in good yields (Chart 1). Table I shows the results of asymmetric reductions by both reagents.



1 2

	R_f	R	Y(%) of 1
a	CF_3	nC_6H_{13}	84
b	CF_2H	nC_5H_{11}	79
c	CFH_2	nC_5H_{11}	66

* R-BINAL-H or S-Alpine-Borane

Chart 1

Table I. Enantioselective Reduction of 1

Compound	Reagent[a]	Yield(%)	% ee of 2[b]	Config.[c]	$[\alpha]_D^{23}$/MeOH
2a	A	88	19 (21)	R	-0.76 (c=0.52)
	B	79	75 (90)	R	-2.85 (c=0.42)
2b	A	97	58 (65)	S	-3.10 (c=1.09)
	B	94	75 (90)	R	+3.80 (c=1.05)
2c	A	96	65 (73)	S	+12.08(c=1.27)
	B	99	62 (74)	R	-11.62(c=1.29)

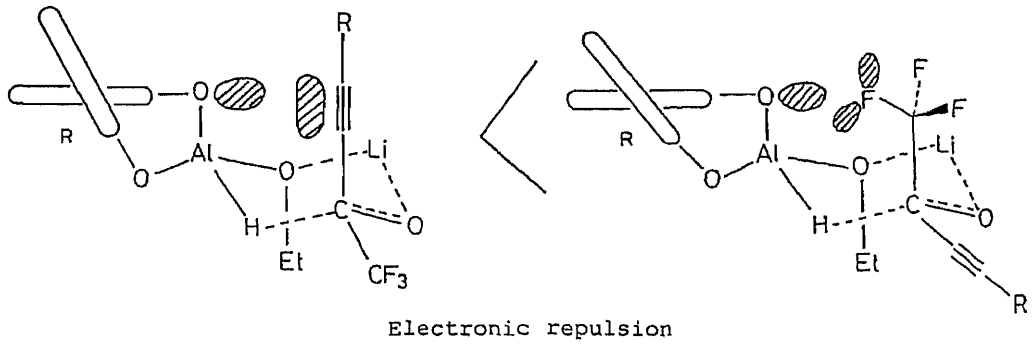
a) A: (R)-BINAL-H was prepared in THF from $LiAlH_4$, EtOH (1 eq) and (R)-binaphthol (1 eq)(88% ee). Reaction Conditions; 3eq. of BINAL-H , -100°C, 1 h.
B: (S)-Alpine-Borane (83% ee) (2 eq.) in THF, Reaction Conditions; room temp., overnight.

b) Determined by ^{19}F- and ^{1}H-NMR analysis of the (-)-MTPA-ester of 2. The numbers in parentheses are corrected for 88% ee binaphthol and 83% ee Alpine Borane.

c) See ref. 7.

In the reduction of (R)-BINAL-H, the lowering of the optical yield in the product (2a-c) by increasing the number of fluorine atoms of the starting ketones (1a-c) indicates that the electronic repulsion between the fluorine atom(s) and the lone pair of binaphthoxyl oxygen is significant. It should be noted that the absolute configuration of the trifluoromethyl compound (2a, R) differs from those of the difluoro- and monofluoromethyl compounds (2b and 2c, S) and the poor optical purity (19% ee) of 2a. The absolute configurations of 2b and 2c can be rationalized on the basis of the transition state for BINAL-H reduction as in the reported examples.[6] From these findings, it is evident that the electronic repulsion between the electronegative trifluoromethyl group and the lone pair of binaphthoxyl oxygen of the reagent overrides the electronic repulsion between the

π-electrons of the triple bond and the lone pair of binaphthoxyl oxygen (Chart 2). Although the steric factors of the trifluoromethyl groups are also of some significance, the bulk of the trifluoromethyl group is not a major factor in changing the enantioselectivity of the BINAL-H reagent, since the iso-propyl group with steric bulk comparable to that of the trifluoromethyl group[8] has been reported not to alter the enantioselectivity of BINAL-H.[6] To confirm the electronic effect of the trifluoromethyl group, trifluoromethyl n-octyl ketone (3) was reduced with (R)-BINAL-H. The chirality (R) and optical purity (61% ee) of the alcohol (4)[7] indicated that the trifluoromethyl group functions effectively as a repulsive component in an electronic sense. The notably different character of the trifluoromethyl group from that of the difluoro- and monofluoromethyl groups could also be attributed to the pseudo-π character of the trifluoromethyl group.[6,9]



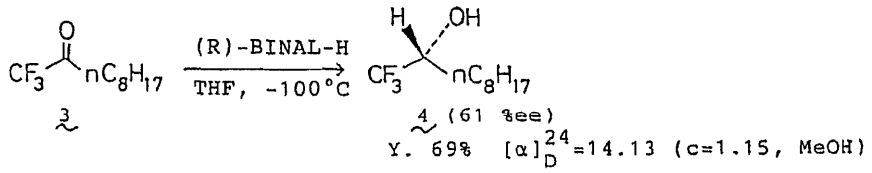
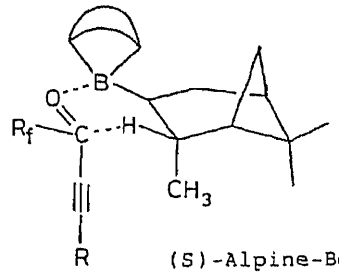


Chart 2

In the (S)-Alpine-Borane reduction, all the fluoroalkyl alkynyl ketones (1a-c) gave alcohols (2a-c) of the anticipated absolute configuration in reasonable optical yields (62-75 %ee). As mentioned above, the purely steric effect is important in Alpine-Borane reduction. It is quite apparent that the fluoroalkyl groups behave in the same way as the alkyl groups to avoid 1,3-diaxial interaction in the cyclic transition state in which the carbon-carbon triple bond is the least steric requirement.[5]



(S)-Alpine-Borane

In the enantioselective reduction of fluoroalkyl alkynyl ketones, the strong electronic effect of the trifluoromethyl group exerted influence on the enantioselectivity of the BINAL-H reagent. On the basis of these findings, we

prepared a series of fluoroalkylated sugar derivatives in optically active form. This will be published elswhere in due course.

Acknowledgement: This work was supported in part by a grant from the Toray Foundation for the promotion of Science and Technics.

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Communications to the Editor

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TWO NEW ANTINEOPLASTIC DITERPENES FROM TAXUS MAIREI[1)]

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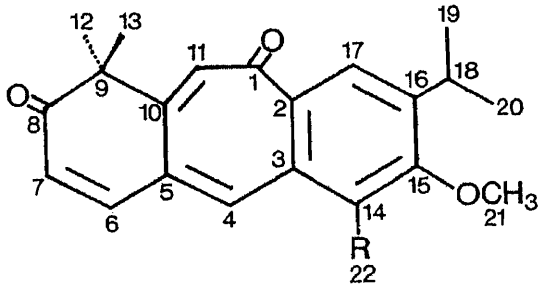
Two new diterpenes, taxamairin A and B, were isolated from the bark of Taxus mairei. Their structures were determined by spectroscopic means, and finally confirmed by X-ray analysis.

KEYWORDS —— Taxus mairei; Taxaceae; diterpene; taxamairin A; taxamairin B; antineoplastic activity

Taxus mairei (Lemeé et Lévl.) S. Y. Hu (Taxaceae) grows in Fujiang province, China.[2)] From the ethanol extract of the bark, two new diterpenes, named taxamairins A and B, possessing a tropone skeleton were isolated together with some taxane-type diterpenes.[3)] These new compounds displayed inhibitory activity (IC_{50} 30.21 μg/ml (taxamairin A) and 26.78 μg/ml (taxamairin B)) against hepatoma cells in vitro. The structure of these compounds was determined by spectroscopy, and finally confirmed by X-ray analysis. To the best of our knowledge, this paper is the first report of diterpenes having a novel skeleton as natural products.



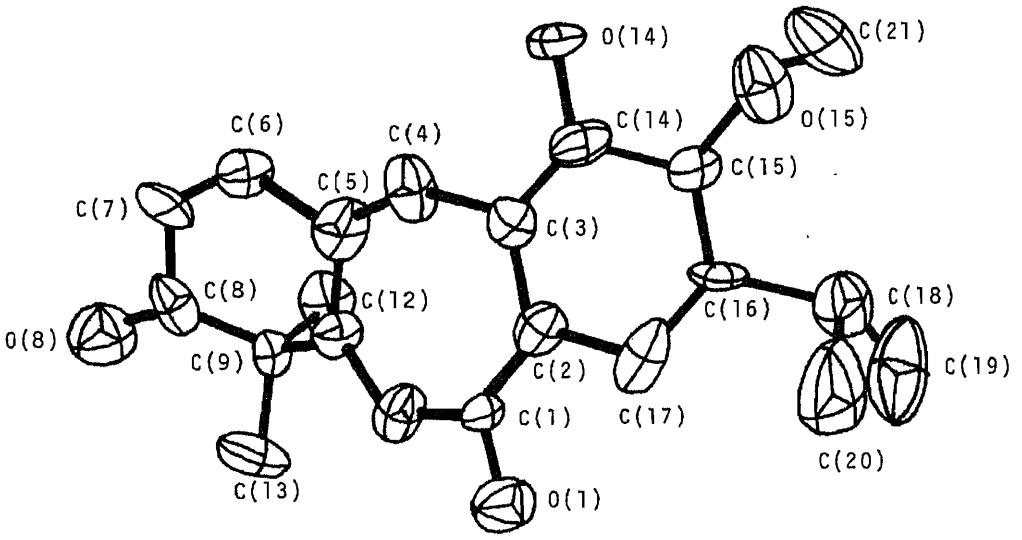
1: R=OH taxamairin A

2: R=OCH3 taxamairin B

Taxamairin A (1) (0.02% for total extract) was obtained as white crystals from EtOH, mp 223-224°C, MW 338.1505 (Calcd. 338.1518), $C_{21}H_{24}O_4$, CD (MeOH) $\Delta\varepsilon = 0$, UV λ_{max}^{EtOH} nm (log ε): 211 (4.43), 255 (4.46), 385 (4.34), ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.33 (6H, d, J= 6.4 Hz, 19 and 20-CH_3), 1.46 (6H, s, 12 and 13-CH_3), 3.36 (1H, heptet, J= 6.46 Hz, 18-H), 3.99 (3H, s, 21-OCH_3), 6.11 (1H, d, J= 10.0 Hz, 7-H), 6.44 (1H, br s, 14-OH), 6.94 (1H, s, 11-H), 7.30 (1H, d, J= 10.0 Hz, 6-H), 7.77 (1H, s, 4-H), IR ν_{max}^{KBr} cm^{-1}: 3410, 1676, 1622. The crystals of 1 used for X-ray analysis were recrystallized from MeOH. The crystals are a monoclinic system, space group C_{2h}^5-P^{21}/C with four molecules

in the unit cell. The cell dimensions are a= 10.0907 Å, C= 21.7957 Å, b= 8.2893 A, β= 96.744°, v= 1810.48 Å^3, D_c= 1.23 g/cm^{-1}, F(000)= 704 e, $\mu_{MO}K_\alpha$= 0.9 cm^{-1}, Z= 4. The X-ray analysis showed that all molecules of 1 were on a plane, except C-9, 10 and 11 which appeared only a little twisted. It could be explaied that the result of CD of 1 was zero.

Taxamairin B (2) (0.0025%) was obtained as white crystals, MW 352.1626 (Calcd. 352.1675), $C_{22}H_{24}O_4$, CD (MeOH) $\Delta\varepsilon$ = 0, UV λ_{max}^{EtOH} nm (log ε): 219 (4.10), 281 (4.12), 355 (3.94), ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.29 (6H, d, J= 6.4 Hz, 19 and 20-CH_3), 1.44 (6H, s, 12 and 13-CH_3), 3.40 (1H, heptet, J= 6.4 Hz, 18-H), 3.95 (3H, s, 22-OCH_3), 3.98 (3H, s, 21-OCH_3), 6.11 (1H, d, J= 10.0 Hz, 7-H), 6.92 (1H, s, 11-H), 7.30 (1H, d, J= 10.0 Hz, 6-H), 7.85 (1H, s, 4-H), 7.92 (1H, s, 17-H), IR ν_{max}^{KBr} cm^{-1}: 1675, 1640, 1610. Except for the number of methoxy groups, the ^{1}H-NMR spectrum of 2 was very similar to that of 1. The spectral data for the compound (2'), methylated derivative of taxamairin A (dimethyl sulfate, K_2CO_3 in acetone), were in good agreement with those for 2, and the melting point of 2' was not depressed in a mixture with 2.



ACKNOWLEDGEMENT The authors are grateful to Prof. Xu. Xiao-jie (Faculty of Chemistry, Benjing University, Benjing, China) for the X-ray analysis.

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Communications to the Editor

[Chem. Pharm. Bull. 35(6)2615—2617(1987)]

STRUCTURES OF TOROKONINE AND GOMANDONINE, TWO NEW DITERPENE ALKALOIDS FROM ACONITUM SUBCUNEATUM NAKAI

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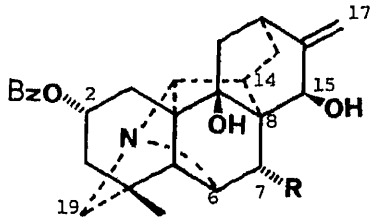
Chemical investigation of the roots of Aconitum subcuneatum Nakai resulted in the isolation of two new diterpene alkaloids torokonine (1) and gomandonine (2), along with eight known compounds, i.e. mesaconitine, jesaconitine, 14-dehydrobrowniine, neoline, 15-α-hydroxyneoline, isotalatizidine, senbusine A, and virescenine. The structure and the absolute configuration of torokonine (1) were established as 7-α-hydroxyryosenamine from the spectroscopic analysis. The novel structure of gomandonine (2), having an epoxy ring, was initially deduced by the pyridine-induced solvent shift technique and then confirmed by X-ray analysis.

KEYWORDS—— Aconitum Subcuneatum; Ranunculaseae; diterpene alkaloid; torokonine; gomandonine; ^{13}C-NMR; CD; solvent shift; X-ray analysis

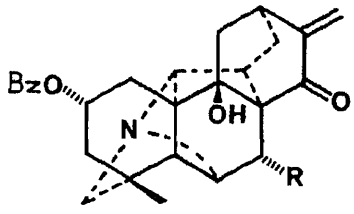
We have determined the structure of two new diterpene alkaloids, named torokonine and gomandonine isolated from the roots of Aconitum subcuneatum Nakai collected at Mt. Gomando, Fukushima prefecture, in July 1982.

Torokonine (1), which was originally isolated from the same plants and was named Gomando-base I by Ochiai et al.,[1] has the following physical constants: mp 198.5-199°C (from acetone), $C_{27}H_{31}NO_5$, (m/z; M^+ Calcd 449.2202, Found 449.2234), $[\alpha]_D^8$ +71.7°(c=0.14, MeOH), IR(KBr);3400, 1720, ^{1}H-NMR(CD_3OD):δ 7.47-8.02(5H, aromatic H), 5.52(1H, m, C_2-H), 5.03 and 5.00(each 1H, t, J=1.3Hz, C_{17}-H_2), 4.53(1H, br s, C_{15}-H), 4.42(1H, d, J=2.6Hz, C_7-H), 3.41(1H, br s, C_6-H), 3.11 and 2.60(each 1H, d, J=12.5Hz, C_{19}-H_2), 2.96(1H, s, C_{20}-H), 1.11(3H, s, C_{18}-H_3). These data for torokonine, which are very similar to those of ryosenamine (3),[2] indicate the presence of an additional secondary hydroxy group in ryosenamine molecule. In the ^{13}C-NMR spectrum (Table I), the appearance of a new doublet at 64.3 ppm, the absence of a triplet at 29.1 ppm (compared with ryosenamine (3)), and the downfield shift at C_6 (5.6 ppm) and at C_8 (4.8 ppm) indicate that an extra secondary hydroxy group must be present at the C_7 position. Furthermore, upfield shifts of C_{14} (6.0 ppm) and of C_{15} (5.9 ppm) due to steric compression by the OH function give proof of the α-orientation of the C_7 hydroxy group. The absolute configuration of torokonine (1) was determined as follows. 1 was oxidized with active MnO_2 in CH_2Cl_2 at room temperature to yield α,β-unsaturated ketone

derivative (4), mp 248-250°C(from MeOH), IR(KBr):3540, 1715, 1645, ^{1}H-NMR($CDCl_3$)δ: 5.85, 5.10(each 1H, d, J=1Hz, C_{17}-H_2). The CD spectra of 4 [$\lambda_{ext}^{dioxane}$ nm($\Delta\epsilon$): +0.82(362), +0.82(349), +14.0(235.5)] and that of ryosenaminone (5)[2] [$\lambda_{ext}^{dioxane}$ nm($\Delta\epsilon$): +0.53(363), +0.57(348), +14.30(235)] showed similar Cotton curves. Thus, the absolute configuration of 1 is identical with that of ryosenamine (3) having (-)-kaurene type. We also made it clear that an unidentified alkaloid tentatively named Toroko-base I[1] that has been isolated from Aconitum subcuneatum Nakai collected at Toroko(Akita Prefecture) is torokonine (1) by comparison of the physical and spectral data.



(1) R=OH, torokonine
(3) R=H , ryosenamine



(4) R=OH
(5) R=H

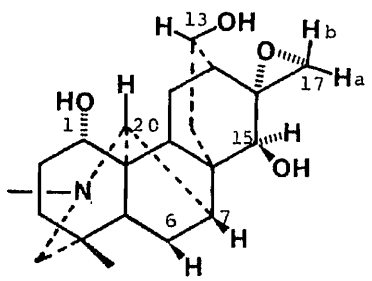
Table I. ^{13}C Chemical Shifts of 1, 3, and 2

Carbon	1	3	2
1	28.9	29.2*	70.6
2	70.2	70.8	32.1
3	39.2	38.8	40.1*
4	35.4	35.9	33.8
5	51.5	54.3	52.7
6	69.7	64.1	24.1#
7	64.3	29.1*	42.6@
8	48.9	44.1	44.6
9	79.6	79.3	43.9@
10	49.8	50.5	51.4
11	36.9	37.2	25.6#
12	34.6	35.0	41.6@
13	32.7	33.6	69.1
14	36.0	42.0	39.2*
15	66.6	72.5	76.5
16	153.6	155.2	65.5
17	110.5	109.6	45.0
18	29.3	29.5	26.3
19	62.2	63.7	59.6
20	73.3	74.2	68.9
C=O	165.9	166.0	
	130.1	130.4	
	129.4	129.4	
	128.8	128.6	
	133.3	133.0	
N-CH_3			43.9

Assignments with the same symbol are interchangeable in each column. δ (ppm) downfield from TMS in $CDCl_3$ 1,3 and in d_5-pyridine 2.

Gomandonine (2), [mp 248-249°C(from MeOH), $[\alpha]_D^{12}$ -42.5°(c=0.12, MeOH), $C_{21}H_{31}NO_4$], has the following spectral data: IR(KBr):3570, 3400, 3310, MS m/z(%):361(M^+, 41), 344(M^+-OH, 100), ^{1}H-NMR(d_5-pyridine+D_2O) δ:5.00(1H, s, C_{15}-H), 4.37(1H, d.d, J_1=4.4HZ, J_2=8.4Hz, C_{13}-H), 4.13(1H, d.d, J_1=6.3Hz, J_2=10.9Hz, C_1-H), 3.89(1H, br s, C_{20}-H), 3.63(1H, d, J=6.3Hz, C_{17}-H_a), 2.66(1H, d, J=6.3Hz, C_{17}-H_b), 3.47(1H, br.d,d, J_1=8.2Hz, J_2=13.8Hz, C_6-βH), 2.25(3H, s, N-CH_3), 0.71(3H, s, CH_3). We have deduced from the above physico-chemical data that gomandonine is a C_{20} type diterpene alkaloid having one N-CH_3 group and three secondary hydroxy groups in the molecule. But most characteristic of this new compound is the absence of the exomethylene group that always exists in the hitherto known C_{20} diterpene alkaloids. ^{13}C-NMR spectrum analysis (Table I) indicated the presence of an epoxy ring at the C_{16}-C_{17} position. Initially, we deduced the location and the stereochemistry of the three hydroxy groups (C_1-OH, C_{15}-OH, and C_{13}-OH) utilizing

the pyridine-induced solvent shift technique in ^{1}H-NMR spectra.[3] Individual protons (C_{20}-H, C_6-βH, and C_{15}-αH) occupying positions sterically close to the hydroxy function (C_1-αOH, C_{15}-βOH, and C_{13}-OH) shift dramatically downfield (0.41 ppm, 0.77 ppm and 0.69 ppm, respectively) in d_5-pyridine relative to $CDCl_3$. Furthermore, one signal of the protons on C_{17} showed a pyridine-induced solvent shift by -0.51 ppm, a fact which indicates that C_{15}-βOH and C_{17}-H_a have the 1,3-diaxial relationship, and consequently that the epoxy ring orients to the α-side. The structure of 2 indicated by the considerations above was confirmed by X-ray analysis. Crystals of gomandonine (2) belong to a monoclinic space group, $P2_1$, with the cell parameters of a=10.531(3), b=8.074(4), c=11.743(2)Å, β=112.44(2)°, Z=2, and Dx=1.30g/cm^3. The structure was solved by the direct method MULTAN and the result was refined by a block diagonal least squares procedure to R=0.072 for 1905 unique reflections with Fo>3σ(Fo) measured on a Rigaku AFC-5 diffractometer with Cu$K\alpha$ radiation. The ORTEP drawing of the structure of gomandonine (2) is shown in Fig. 1.



(2) gomandonine

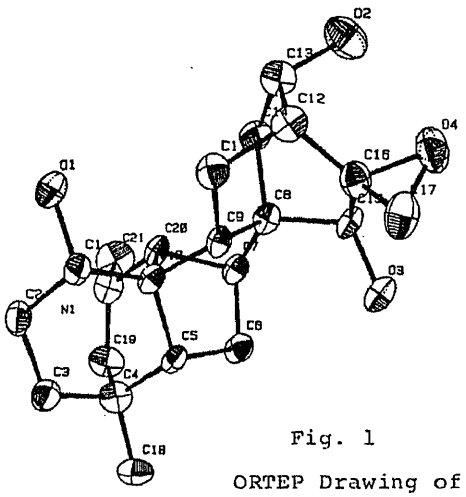


Fig. 1
ORTEP Drawing of (2)

ACKNOWLEDGEMENT The authors are much indebted to emeritus Prof. Toshihiko Okamoto of Tokyo University for his kind encouragement.

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Communications to the Editor

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SYNTHESES OF THE β-ERYTHROIDINE SKELETON[1)]

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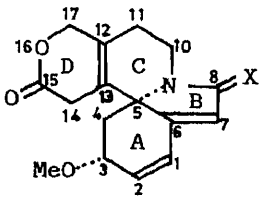
The β-erythroidine skeleton was synthesized by two different routes; one was a four-step synthesis starting from 15,16,17-trimethoxy-8-oxo-*cis*-erythrinan, the other was a five-step synthesis starting from D-furano-8-oxo-*cis*-erythrinan. The two routes gave the same 14,17-dihydro-16(15H)-oxa-erythrinan-8,15-dione (3).

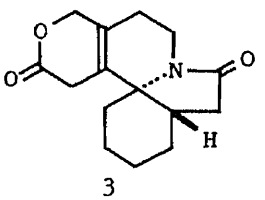
KEYWORDS ——— Erythrina alkaloid; β-erythroidine; ozonolysis; aromatic ring cleavage; photodecarboxylation; D-furano-erythrinan; N-bromosuccinimide oxidation; allylic rearrangement

Among Erythrina alkaloids, β-erythroidine (1)[3)] is particularly interesting in its strong curare like activity on oral administration. Structurally the alkaloid belongs to the non-aromatic type of the erythrinan group. Although many attempts are reported,[4)] the synthesis of those non-aromatic erythrinan alkaloids is still elusive. Here we present the synthesis of β-erythroidine skeleton (3) by two different routes. One route starts from trimethoxyerythrinan (4) and uses a selective ozonolytic cleavage of the aromatic ring and a photochemical removal of an allylic carboxyl group as crucial steps. The other one starts from D-furano-8-oxo-erythrinan (11), converting its furan ring to the unsaturated δ-lactone.



1: X=H_2

2: X=O

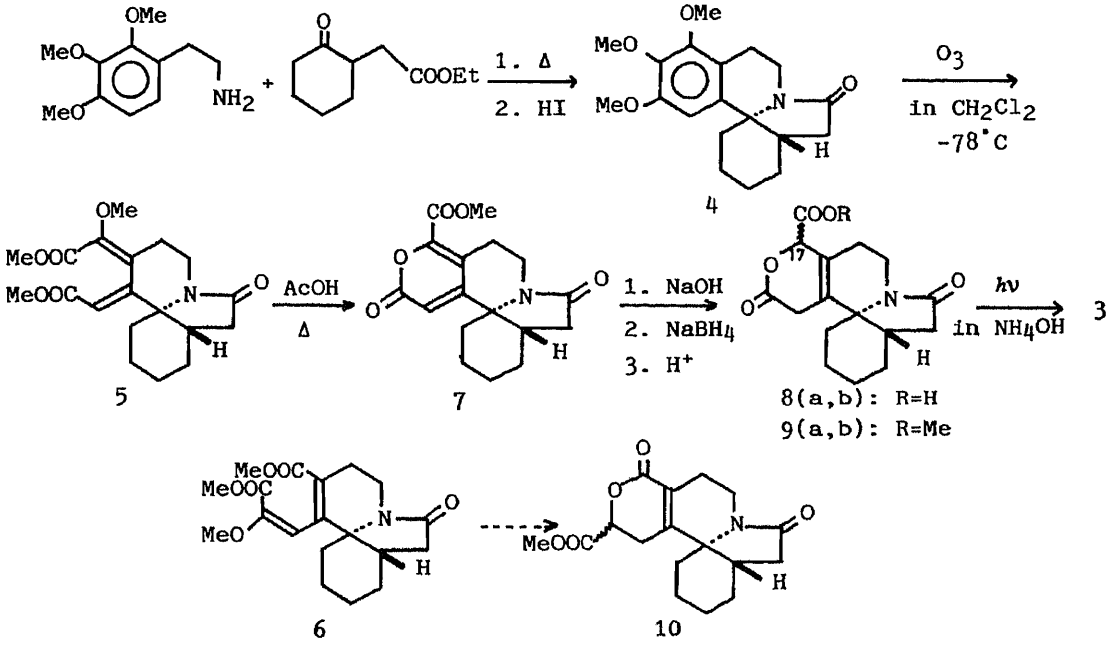


3

15,16,17-Trimethoxy-8-oxo-*cis*-erythrinan (4)[5)] was prepared in 80% yield by condensation of 2,3,4-trimethoxyphenylethylamine and ethyl cyclohexanone-2-acetate followed by the cyclization with 57% hydroiodic acid in toluene at room temperature. Compound 4 was ozonized in CH_2Cl_2 at -78°C for 40 min to give the diester (5) (33%), and its isomer (6) (15%). Their spectral data indicate that both esters are products whose aromatic rings were cleaved between two methoxy groups. The following transformation determined the structure of the major product (5). On heating the

major product (5) with 70% acetic acid at 130°C in a sealed tube, a pyron derivative (7) was produced (57%). Alkaline hydrolysis of 7 with 10% NaOH followed by treatment of the resulting keto-diacid with sodium borohydride gave, after acidification, a mixture of the δ-lactones (8) epimeric at the carboxyl group in 66% yield. This was converted to the methyl esters (9) on treatment with diazomethane in methanol, and separated into the epimers (**9a**) (mp 117-119°C) and (**9b**) (oil) by preparative HPLC. In the ^{1}H-NMR spectra both epimers showed C_{17}-H as a broad singlet (δ 5.07 for **9a** and δ 5.04 for **9b**), thus confirming the structure 9. The corresponding methyl ester (10) expected from the isomer **6** should show a triplet or a double doublet signal for this hydrogen.

The carboxyl group at the allylic position in 8 was removed photochemically.[6] Irradiation of 8 (mixture of **8a** and **8b**) in 3N NH_4OH with a 100 W high pressure mercury lamp (without filter) at room temperature for 2 h gave the decarboxylated product (3) in 18% yield. The ^{1}H-NMR signals of ring D of this compound were in good agreement with those of natural 8-oxo-β-erythroidine (2).[7] The ^{13}C-NMR also supported the assigned structure.[8]



For further confirmation of the structure 3, an alternate synthesis of 3 was undertaken starting from D-furano-8-oxo-*cis*-erythrinan (11).[9]

Treatment of the D-furano-erythrinan (11) with 3 eq mol of N-bromosuccinimide in H_2O-dioxane (1:5) yielded the hydroxy-γ-lactone (12) (72%), which on hydrogenation over 10% Pd/C in ethanol afforded an epimeric mixture of keto-acids (13). The epimers were separated by converting the mixture into the methyl esters **14a** (61%) and **14b** (30%).[10] The less polar ester (**14a**, mp 93-96°C) was stable to sodium methoxide in methanol, while the more polar ester (**14b**, mp 83-85°C) was completely changed into **14a** on treatment with 5% sodium methoxide in methanol at room temperature, thus proving that they are the stereoisomers.

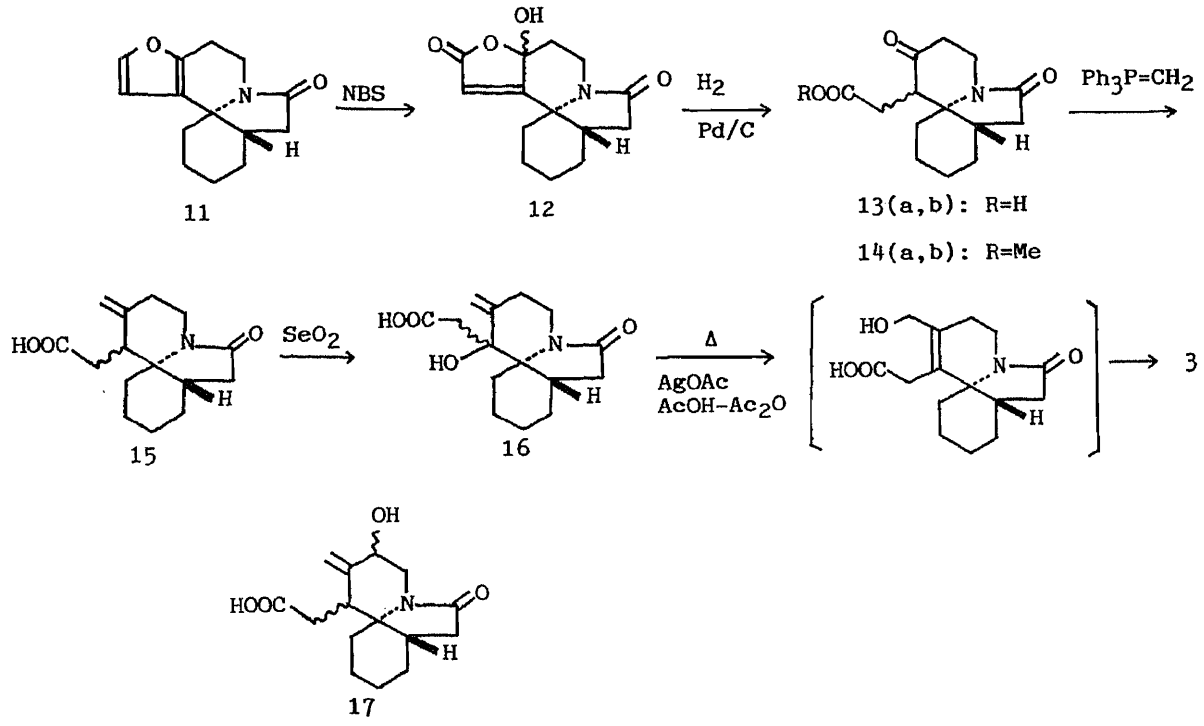
The Wittig reaction of a mixture of the keto-acids (13) with methylenetriphenyl-

phosphorane in toluene gave the exo-methylene derivative (15) as a sole product (93%). Selenium dioxide oxidation of 15 in the presence of silver acetate in EtOH-H_2O (5:1) under reflux for 5 h gave two hydroxy compounds, 16 (50%) and (17) (49%), which were characterized respectively by the absence and presence of a hydrogen geminal to the hydroxyl group in their ^{1}H-NMR spectra.

Finally, heating the allylic alcohol (16) in AcOH-Ac_2O with silver acetate (or tetrabutylammonium acetate) at 100°C (sealed tube) effected an allylic rearrangement and cyclization of the resulting primary alcohol, giving rise to the compound (3) in 21% yield, which was identical with the above-described sample in all respects.

Previously, Kitahara and Matsui[11] reported the synthesis of a compound which they thought had the structure 3. However the reported physical data, particularly the ^{1}H-NMR [mp 155-156°C; IR: 1725, 1685, 1620; δ 4.65 (2H, s)], are quite different from those of ours. Although direct comparison of the two samples was not feasible because the previous sample has been lost, they are apparently different.

We therefore believe that the present synthesis provides the first synthesis of the β-erythroidine skeleton.



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5) All new compounds in this communication had satisfactory spectral data. The IR specra were taken in KBr or $CHCl_3$ and given in cm^{-1}. The ^{1}H-NMR and ^{13}C-NMR spectra were at 100 MHz and given in δ. High resolution MS were measured at 30 eV.

4: mp 91-92°C; $C_{19}H_{25}NO_4$; IR 1685; ^{1}H-NMR 6.69 (1H, s, ArH), 3.86 (9H, s, 3xOCH_3).

5: mp 112-113°C; $C_{19}H_{25}NO_6$; IR: 1735, 1695, 1655; ^{1}H-NMR: 5.88 (1H, s, olefinic H), 3.72 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 3.67 (3H, s, OCH_3).

6: Oil; $C_{19}H_{25}NO_6$; IR: 1730, 1670; ^{1}H-NMR: 5.66 (1H, s, olefinic H), 3.74 (6H, s, OCH_3), 3.64 (3H, s, OCH_3).

7: mp 156-158°C; $C_{17}H_{19}NO_5$; IR: 1715, 1700, 1670; ^{1}H-NMR: 6.57 (1H, s, olefinic H), 3.92 (3H, s, OCH_3).

9a: Oil; $C_{17}H_{21}NO_5$; IR: 1765, 1740, 1700; ^{1}H-NMR: 5.07 (1H, br s, C_{17}-H), 3.78 (3H, s, OCH_3), 3.28 (2H, br s, C_{14}-H).

9b: Oil; $C_{17}H_{21}NO_5$; IR($CHCl_3$): 1760, 1740 (sh), 1680; ^{1}H-NMR: 5.04 (1H, br s, C_{17}-H), 3.82 (3H, s, OCH_3), 3.27 (2H, br s, C_{14}-H).

11: mp 117.5-119°C; $C_{14}H_{17}NO_2$; IR: 1680; ^{1}H-NMR: 7.29 (1H, d, J=1.8 Hz, furan H), 6.44 (1H, d, J=1.8 Hz, furan H).

3: mp 190°C (dec); $C_{15}H_{19}NO_3$; IR: 1755, 1725, 1620; ^{1}H-NMR: 4.72 (1H, d) and 4.63 (1H, d, J=15.6 Hz, OCH_2C=C), 4.23(1H, dd, J=13.3, 7.1 Hz) and *ca.* 2.96 (1H, m, C_{10}-H_2), 3.16 (2H, br s, O=CCH_2C=C).

12: mp 228.5-230.5°C; $C_{14}H_{17}NO_4$; IR: 3275, 1770, 1675; ^{1}H-NMR: 5.91 (1H, s, C=CH).

14a: mp 93-96°C; $C_{15}H_{21}NO_4$; IR: 1740, 1712, 1685; ^{1}H-NMR: 3.69 (3H, s, OCH_3).

14b: mp 83-85°C; $C_{15}H_{21}NO_4$; IR: 1745 (sh), 1735, 1700; ^{1}H-NMR: 3.69 (3H, OCH_3).

15: mp 180-183°C; $C_{15}H_{21}NO_3$; IR: 1710, 1630; ^{1}H-NMR: 5.02, 4.81 (each 1H, s, C=CH_2).

16: mp 195-208°C (dec); $C_{15}H_{21}NO_4$; IR: 3350, 1700, 1620; ^{1}H-NMR: 5.00, 4.86 (each 1H, s, C=CH_2).

17: mp 180-183°C; $C_{15}H_{21}NO_4$; IR: 3360, 1700, 1630; ^{1}H-NMR: 5.15, 4.89 (each 1H, s, C=CH_2), 4.20 (1H, br s, HOC$\underline{H}$<).

6) K. Isobe, J. Taga, Y. Toyokawa, and Y. Tsuda, to be published. *Cf.* The 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, 1976.

7) 8-Oxo-β-erythroidine (2): IR: 1750, 1700; ^{1}H-NMR: 4.73, 4.59 (each 1H, d, J=16.0 Hz, OCH_2C=C), 3.10 (2H, s, $OCCH_2C$=C) [A. S. Chawla, A. H. Jackson and P. Ludgate, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 2903].

8) ^{13}C-NMR: 174.1, 168.9 (s, C=O), 130.8, 124.4 (s, C=C), 70.5 (t, $O\underline{C}H_2C$=C), 61.5 (s, -$\underline{C}$-), 34.4 (d, -$\underline{C}$H), 36.3, 33.3, 31.3, 30.7, 26.9, 24.0, 19.8, 19.7 (t, >CH_2). *Cf.* A. S. Chawla, S. Chunchatprasert and A. H. Jackson, *Org. Magn. Reson.*, **21**, 39(1983).

9) This was prepared from the reported compound (**18**) [Y. Tsuda, Y. Sakai, M. Kaneko, Y. Ishiguro, K. Isobe, J. Taga, and T. Sano, *Heterocycles*, **15**, 431 (1981)]. Details will be reported in a full publication.

10) Neither **14a** nor **14b** was identical with the compound assigned as **14** by Kitahara and Matsui[11] in their synthesis. IR: 1735, 1720 (sh), 1680; ^{1}H-NMR: 3.86 (3H, s, OCH_3).

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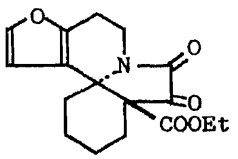

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