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Adsorption of Dodecylammonium Ion on Hydroxyapatite and Concurrent Release of Phosphate and Calcium Ions from the Surface of Hydroxyapatite[1]

SABURO SHIMABAYASHI,*,[2] HIDEJI TANAKA, and MASAYUKI NAKAGAKI

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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Concentrations of phosphate ion ($[Pi]_f$) and calcium ion ($[Ca^{2+}]_f$), liberated from the surface of synthetic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAP) during the adsorption of dodecylammonium chloride (DAC), were determined as a function of equilibrium concentration of dodecylammonium ion ($[DA^+]_f$). When $[DA^+]_f$ was lower than its critical micelle concentration (cmc), $[Ca^{2+}]_f$ increased with increase in $[DA^+]_f$ owing mainly to ion-exchange between dodecylammonium ion (DA^+) and calcium ion (Ca^{2+}) on the surface of HAP. However, $[Pi]_f$ decreased with increase in $[Ca^{2+}]_f$ to keep the solubility product for HAP (K_{sp}) constant. On the other hand, when $[DA^+]_f$ was higher than the cmc, $[Pi]_f$ increased with increase in $[DA^+]_f$ due to the binding of phosphate ion (Pi) to DA^+ micelles. Cationic species (*i.e.*, Ca^{2+} and DA^+) were concomitantly liberated from the surface of HAP to maintain the electroneutrality of the surface phase. Therefore, $[Ca^{2+}]_f$ increased and the adsorbed amount of DA^+ (X_{DA^+}) decreased with increase in $[DA^+]_f$. When $CaCl_2$ was added to the solution, X_{DA^+} decreased with increasing concentration of added $CaCl_2$ owing to the competitive adsorption between DA^+ and added Ca^{2+}. The release of Pi was strongly depressed because of the electrostatic interaction between surface Pi and adsorbed Ca^{2+}, and because of the requirement of constant K_{sp} in the bulk solution.

Keywords——hydroxyapatite; dodecylammonium chloride; dodecylammonium ion; adsorption; ion-exchange; phosphate ion release; calcium ion release; solubility product; micelle; competitive adsorption

Introduction

Hydroxyapatite (HAP) is a basic calcium phosphate which has the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. In the human body, HAP exists as the major inorganic constituent of calcified tissues (bone and teeth) and as the major or minor component of renal calculi. Therefore, knowledge of the surface properties of HAP is of great importance.

In the previous studies,[3,4] the influence of anionic surfactant on HAP surface was examined. It was shown that calcium ion (Ca^{2+}) and/or phosphate ion (Pi) were released from the surface of HAP during the adsorption of dodecyl sulfate ion (DS^-) on HAP. The ratio of these constituent ions released from HAP was nonstoichiometric (*i.e.*, $Ca^{2+}/Pi = 10/6$) and depended on various factors, such as the adsorbed amount of DS^-, the concentration of DS^-,

and the species and concentration of added salt.

In the present work, the influence of dodecylammonium chloride (DAC), a cationic surfactant, on the HAP surface was studied at constant mixing ratio of HAP to an aqueous solution of DAC in the presence or absence of added $CaCl_2$. The amounts of calcium and phosphate ions liberated from the surface of HAP were determined as a function of the concentration of dodecylammonium ion (DA^+) and/or the adsorbed amount of DA^+. The results are discussed taking into consideration ion-exchange between DA^+ and calcium ion, binding of phosphate ion to DA^+ micelles, and the solubility product for HAP.

Experimental

Materials——HAP was the same sample as that used in the previous studies.[3,4] DAC, purchased from Tokyo Chemical Industry Co., Ltd., was of extra pure reagent grade and was recrystallized twice from ethanol–ethyl ether mixture (1 : 5).[5] The critical micelle concentration (cmc) of this sample in aqueous solution was found to be 13.8 mM at 30 °C by surface tension measurement using the ring method. Calcium chloride ($CaCl_2$), purchased from Wako Pure Chemical Industries, Ltd., was of reagent grade and was used without further purification.

Methods——HAP was suspended in a DAC solution of known concentration at 30 °C with or without $CaCl_2$, and the suspension was shaken vigorously at frequent intervals. No buffer solutions were used in order to avoid the effect of buffering agents on the properties of the HAP surface and DA^+ micelles. After at least 14 d, which was sufficient to attain adsorption and dissolution equilibria, the suspension was filtered through a Millipore filter with a pore size 0.1 μm, and the filtrate was used for chemical analyses. The adsorbed amounts of DA^+ and Cl^- were calculated from the difference of the concentrations before and after addition of HAP.

The concentration of DA^+ was determined by the methyl orange method.[6] Dodecylammonium ion was allowed to react with methyl orange in a McIlvaine buffer (pH 4.6). The reaction product was extracted with chloroform, and its absorbance was measured at 433 nm on a Shimadzu model UV-180 spectrophotometer.

The concentration of Cl^- was calculated from the activity of Cl^- determined by using an Orion chloride-sensitive electrode (94-17), taking the ionic strength of the solution into consideration.[7] The electrode was connected to an Orion Microprocessor Ionalyzer (model 901) and was calibrated with an aqueous solution of NaCl. Prior to the measurement, DA^+ was removed with cation-exchange resin (Amberlite IR-120B) because it has an interfering effect on the membrane of the Cl^--electrode.

The concentration of Pi released was determined according to the method of Gee *et al.*[8] Phosphate ammonium molybdate complex formed was reduced with stannous chloride. The absorbance of the resulting color was measured at 720 nm after 15 min. Prior to the determination, a large excess of Na_2SO_4 was added to the sample solutions to precipitate DA^+ as a sulfate salt. The precipitate was then removed with a Millipore filter (0.22 μm pore size), and the filtrate was used for the determination. A calibration curve was obtained by using an aqueous solution of K_2HPO_4 with the same treatment as that for the sample solutions.

The concentration of Ca^{2+} was determined by ethylenediaminetetraacetic acid (EDTA) chelatometry with 1-(1-hydroxy-2-naphthylazo)-6-nitro-2-naphthol-4-sulfonic acid (BT indicator) at pH 10. Since DA^+ interferes with the color change of the indicator, a slight excess of sodium dodecyl sulfate was added to the sample solutions prior to the determination in order to precipitate DA^+. The pH of the filtrate was measured on a Toa HM-5ES pH meter.

Precipitate formation of dodecylammonium phosphate at 30 °C was studied by adding an aqueous solution of K_2HPO_4 to that of DAC. Formation of the precipitate was observed visually. The precipitation boundary curve was obtained from the lowest concentration of phosphate which could form the precipitate of dodecylammonium phosphate when it was mixed with the DAC solution of known concentration.

Results

Adsorption Isotherms of DA^+ on HAP

Figure 1A shows the adsorption isotherms of DA^+ on HAP from an aqueous solution of DAC, where all the experimental points lie almost on one curve irrespective of the mixing ratio of HAP to DAC solution (solid/solution ratio). The adsorbed amount of DA^+ (X_{DA^+}) increased to a maximum, and then decreased slightly with increase in the concentration of free DA^+ ($[DA^+]_f$).

Figure 1B shows the adsorption isotherms of DA^+ on HAP, where each curve indicates the isotherm at a constant concentration of $CaCl_2$ added ($[CaCl_2]_i$). When $[DA^+]_f$ was low, X_{DA^+} increased with increase in $[DA^+]_f$ and $[CaCl_2]_i$. However, when the concentration

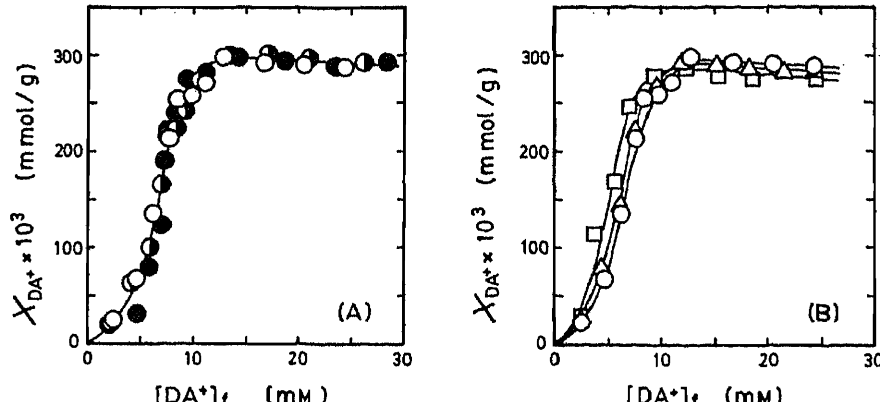


Fig. 1. Adsorption Isotherms of DA^+ on HAP from an Aqueous Solution of DAC in the Absence (A) or Presence (B) of Added $CaCl_2$

(A) Solid/solution ratio (g/l): ○, 20; ◑, 30; ●, 40.
(B) Solid/solution ratio (g/l) = 20.
Initial concentration of added $CaCl_2$, $[CaCl_2]_i$ (mM): ○, 0; △, 2.57; □, 10.3.

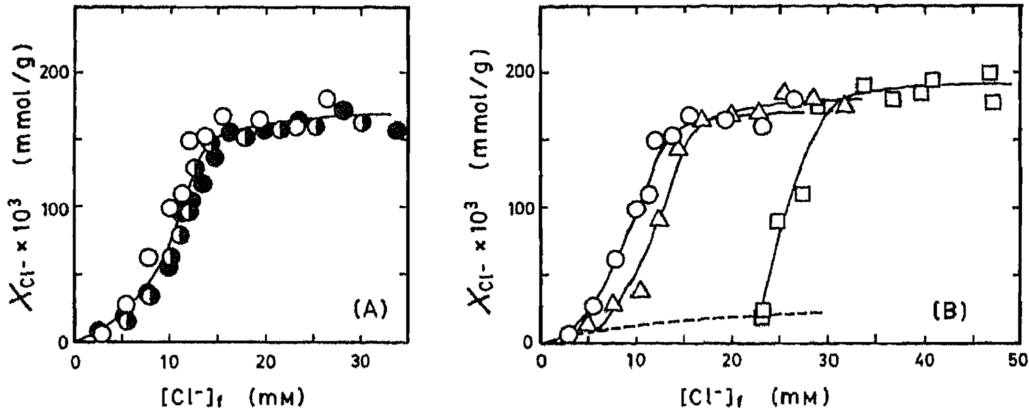


Fig. 2. Adsorption Isotherms of Cl^- on HAP from an Aqueous Solution of DAC in the Absence (A) or Presence (B) of Added $CaCl_2$

All the symbols and the experimental conditions are the same as in Figs. 1A and B. The dotted line in Fig. 2B shows the adsorption isotherm of Cl^- on HAP from an aqueous solution of $CaCl_2$.

became high, X_{DA^+} decreased with increase in $[DA^+]_f$ and $[CaCl_2]_i$.

Adsorption Isotherms of Cl^- on HAP

Figures 2A and B show the adsorption isotherms of Cl^- on HAP from an aqueous solution of DAC. In the absence of $CaCl_2$ (Fig. 2A), the adsorbed amount of Cl^- (X_{Cl^-}) increased monotonously with increasing concentration of free Cl^- ($[Cl^-]_f$). On the other hand, when $CaCl_2$ was added to the solution (Fig. 2B), the isotherms became rather complex because Cl^- in the solution is supplied from both DAC and $CaCl_2$. The dotted line in Fig. 2B shows the adsorption isotherm of Cl^- from an aqueous solution of $CaCl_2$ (*i.e.*, [DAC] = 0). Therefore, the deviation of the full line from the dotted line shows the effect of the presence of DAC.

Concentration of Ca^{2+}

Figures 3A and B show the relationship between $[DA^+]_f$ and the concentration of Ca^{2+} ($[Ca^{2+}]_f$). It shows that $[Ca^{2+}]_f$ increased with increase in $[DA^+]_f$ (Figs. 3A and B) and the solid/solution ratio (A). The latter effect indicates that the released amount of Ca^{2+} increased

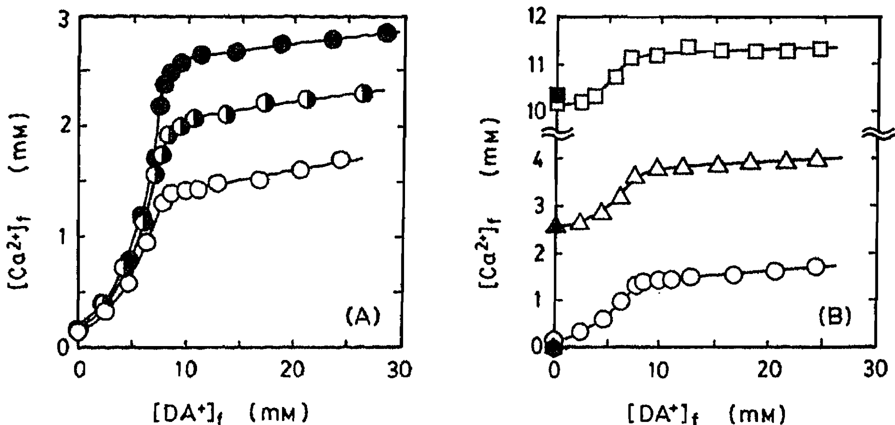


Fig. 3. Relationship between Concentration of Free DA^+ and That of Calcium Ion at Equilibrium in the Absence (A) or Presence (B) of Added $CaCl_2$

All the symbols and the experimental conditions are the same as those in Figs. 1A and B. The closed hexagon (⬢), triangle (▲) and square (■) on the ordinate in Fig. 3B show the initial concentration of Ca^{2+} ($=[CaCl_2]_i$: 0, 2.57 and 10.3 mM).

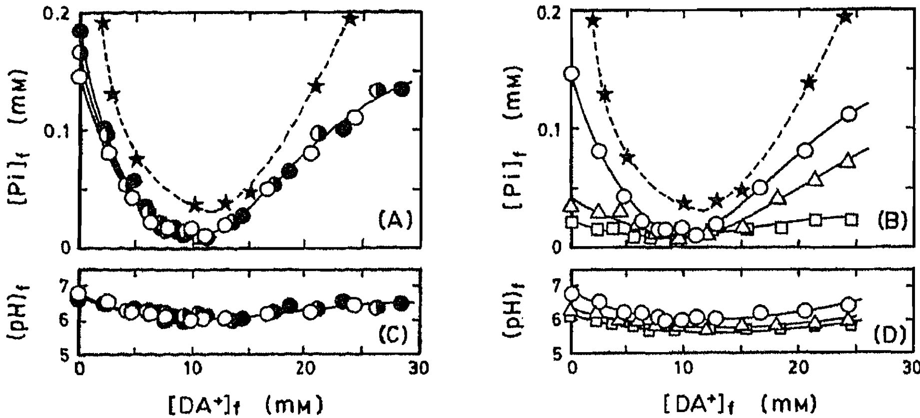


Fig. 4A, B. Relationship between Concentration of Free DA^+ and That of Phosphate Ion Released from the Surface of HAP in the Absence (A) or Presence (B) of Added $CaCl_2$

Fig. 4C, D. Relationship between Concentration of Free DA^+ and Equilibrium pH of the Solution in the Absence (A) or Presence (B) of Added $CaCl_2$

All the symbols and the experimental conditions are the same as in Figs. 1A and B.
---★---: Solubility curve of dodecylammonium phosphate at 30 °C as a function of the concentrations of phosphate and dodecylammonium ions added.

with increasing surface area of HAP added. On the other hand, in the presence of added $CaCl_2$ (B), $[Ca^{2+}]_f$ became lower than the initial concentration of $CaCl_2$ added (shown on the ordinate by the symbol ■, for example), depending on the experimental conditions.

Concentration of Pi and Equilibrium pH of the Solution

The concentration of phosphate ion released from the surface of HAP ($[Pi]_f$) is plotted against $[DA^+]_f$ in Figs. 4A and B. It was found that $[Pi]_f$ decreased to a minimum, and then increased with increase in $[DA^+]_f$. The concentrations of DA^+ at which $[Pi]_f$ has a minimum value are 10.1, 9.9, and 8.3 mM for $[CaCl_2]_i=0$ (○, ◑ and ⬢), 2.57 (△), and 10.3 (□) mM, respectively. The significance of these concentrations is discussed later.

Figures 4C and D show the equilibrium pH, $(pH)_f$, of the solution as a function of $[DA^+]_f$. It was found that $(pH)_f$ decreased or increased in the same manner as $[Pi]_f$ shown in

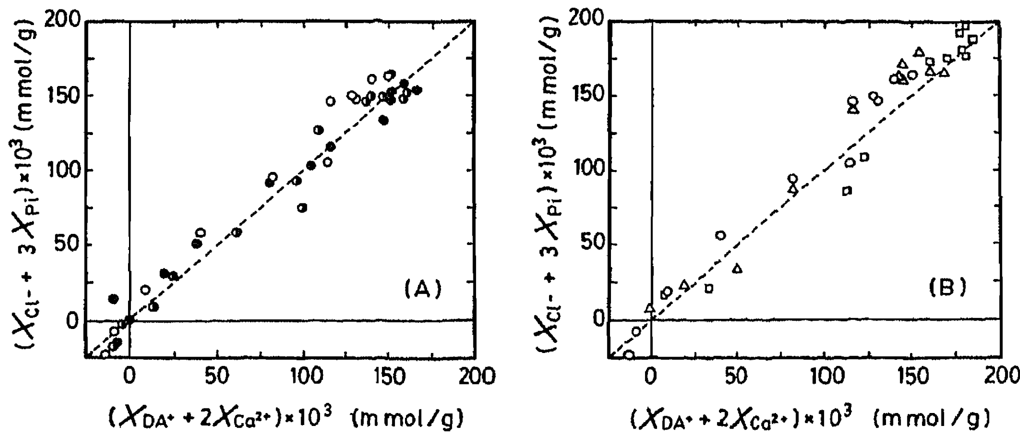


Fig. 5. Relationship between the Increment of Positive Charge and That of Negative Charge on the Surface of HAP at the Adsorption Equilibrium of DAC in the Absence (A) or Presence (B) of Added $CaCl_2$

All the symbols and the experimental conditions are the same as in Figs. 1A and B. The dotted line shows the electroneutrality condition, where $X_{Cl^-}+3X_{Pi}=X_{DA^+}+2X_{Ca^{2+}}$.

Figs. 4A and B. This result suggests that $(pH)_f$ is related to $[Pi]_f$ through the protonation of Pi released into the solution.[3,4] Moreover, the addition of $CaCl_2$ to the solution lowered $(pH)_f$ as shown in Fig. 4D. This tendency has been discussed elsewhere.[9]

Charge Balance at the Surface Phase of HAP

The increase of negative charge at the surface of HAP is plotted against that of positive charge in Figs. 5A and B. The former values were obtained from the amounts of Cl^- adsorption and Pi release, and the latter ones from the amounts of DA^+ adsorption and Ca^{2+} release or adsorption. The symbols X_{Pi} and $X_{Ca^{2+}}$ represent the net increments of Pi and Ca^{2+} at the surface of HAP and were calculated by means of the following equation;

$$X_i = (\text{initial concentration of species i} - \text{equilibrium concentration of species i})/(\text{weight of HAP added}) \quad (1)$$

where i corresponds to Pi or Ca^{2+}. The value of X_{Pi} is negative because, for Pi, only release occurs. On the other hand, $X_{Ca^{2+}}$ has a negative or positive value depending on the experimental conditions. It is known that Pi on the surface of HAP is protonated as a result of hydrolysis.[10] However, in the calculation of the ordinate value, 3 was adopted tentatively as the apparent valency of Pi because the released amount of Pi is very small compared to the other terms and the effect of a change in valency of Pi on most data was within the experimental error; X_{DA^+}, X_{Cl^-} and $X_{Ca^{2+}}$ primarily determine the outcome. As shown in Figs. 5A and B, the slopes are almost unity (see the dotted line): the increase of negative charge is roughly equal to that of positive charge. Hence the electroneutrality of the surface phase of HAP is confirmed.

Discussion

Relationships among the Adsorbed Amount of DA^+ and the Concentrations of Ca^{2+} and Pi Released into the Solution in the Absence of Added Salt

The values of $-\log(Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2$, where ( ) means the activity of the ion in the parenthesis, were obtained according to the method mentioned previously[3,11,12] in the region of $[DA^+]_f < 10.1$ mM, the concentration of DA^+ at the minimum point in Fig. 4A. The numerical values obtained were 113.13—120.69, which are within the range of the literature

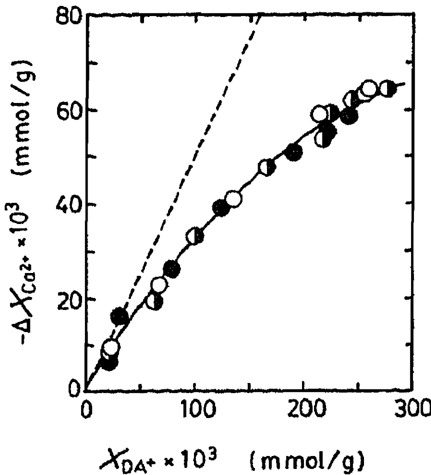
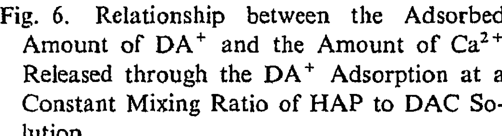


Fig. 6. Relationship between the Adsorbed Amount of DA^+ and the Amount of Ca^{2+} Released through the DA^+ Adsorption at a Constant Mixing Ratio of HAP to DAC Solution


All the symbols are the same as in Fig. 1A. Concentrations of DA^+ corresponding to X_{DA^+} (Fig. 1A) and to $[Ca^{2+}]_f$ (Fig. 3A) for this figure were less than the cmc (10.1 mM). The dotted line shows the hypothetical relationship of $-\Delta X_{Ca^{2+}}/X_{DA^+}=1/2$.

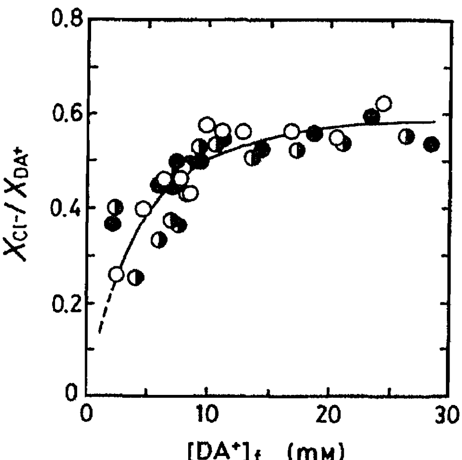


Fig. 7. Relationship between Concentration of Free DA^+ and the Ratio of the Adsorbed Amount of Cl^- to That of DA^+

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

values of $-\log K_{sp}$ for HAP (108—125).[13] This result suggests that the decrease of $[Pi]_f$ in the region of low $[DA^+]_f$ (<10.1 mM) was caused by the increase of $[Ca^{2+}]_f$ to maintain the solubility product for HAP, K_{sp}, constant. On the other hand, Higuchi *et al.* had found that the adsorbed layer of DA^+ retards the release rate of Pi from HAP.[14] Therefore, the adsorption of DA^+ seems to affect both the equilibrium amount of Pi released and the rate of Pi release. Of course, equilibria in the adsorption of DA^+ and the release of Pi were attained in the present work, as mentioned in Experimental.

Figure 6 shows the relationship between X_{DA^+} (quoted from Fig. 1A) and the increment of the released amount of Ca^{2+}, $-\Delta X_{Ca^{2+}}$, due to the addition of DAC in the region of $[DA^+]_f < 10.1$ mM. The latter value was obtained from the data shown in Fig. 3A according to Eq. 2;

$$-\Delta X_{Ca^{2+}} = ([Ca^{2+}]_f \text{ in the presence of DAC} - [Ca^{2+}]_f \text{ in the absence of DAC})/(\text{weight of HAP added}) \qquad (2)$$

It was found that all the experimental points in Fig. 6 are almost on one curve, irrespective of the solid/solution ratio. This result suggests that the increase of $[Ca^{2+}]_f$ with increase in $[DA^+]_f$ in this region is caused by the mechanism of ion-exchange between Ca^{2+} on the surface of HAP and DA^+ adsorbed on HAP. However, the curve deviated downwards from the dotted line (which shows the relationship of $-\Delta X_{Ca^{2+}}/X_{DA^+}=1/2$) as X_{DA^+} increased. The ratio of the released amount of Cl^- to that of DA^+ (X_{Cl^-}/X_{DA^+}) is plotted against $[DA^+]_f$ in Fig. 7. This shows that X_{Cl^-}/X_{DA^+} increased with increasing $[DA^+]_f$. The deviation of the curve in Fig. 6 is, therefore, attributed to the increase of simultaneous binding of Cl^- for the adsorbed DA^+ (Fig. 7) as well as to the decrease of the amount of Pi released (Fig. 4A). A part of DA^+ was adsorbed on HAP through ion-exchange with Ca^{2+} on the surface of HAP (Fig. 6) and the rest was adsorbed together with Cl^- as a counterion (Fig. 7). Thus, the electroneutrality of the surface phase of HAP is maintained, as shown in Fig. 5.

On the other hand, in the region of $[DA^+]_f > 10.1$ mM, $[Pi]_f$ increased with increase in

$[DA^+]_f$ (Fig. 4A), in spite of the increase of $[Ca^{2+}]_f$ in this region (Fig. 3A). As the solubility product for HAP should be constant even in this region, it was concluded that the activity coefficient for PO_4^{3-} decreases with increase in $[DA^+]_f$ owing mainly to the binding of Pi to DA^+ micelles. This conclusion is supported by the fact that $[Pi]_f$ depends only on $[DA^+]_f$, irrespective of the solid/solution ratio. Accordingly, the concentration of 10.1 mM, the minimum point in Fig. 4A, is regarded as the cmc of this system. The positive charge on the surface of HAP resulting from the Pi release is compensated for by the decrease of X_{DA^+} (Fig. 1A), by the increase of X_{Cl^-} (Fig. 2A) and by the release of Ca^{2+} from the surface of HAP. Therefore, Ca^{2+} release continues in the region above the cmc (Fig. 3A), but by a mechanism different from that below the cmc.

The dotted line in Figs. 4A and B is the precipitation boundary curve (PBC) of the phosphate ion–dodecylammonium ion system. It was found that the PBC is V-shaped. Similar diagrams were reported for precipitate formation in systems of metal ion and surfactant anion.[15-19] The region below the PBC is monophasic, whereas dodecylammonium phosphate precipitates in the region above the PBC. The experimental points shown on the full lines in Fig. 4 are obviously in the monophasic region. Therefore, it was concluded that phosphate ion released to the solution from HAP would not precipitate dodecylammonium ion in the solution.

Effect of Added $CaCl_2$

It is known that Ca^{2+} has a higher affinity for HAP than other metal ions.[20] This is because Ca^{2+} is one of the lattice ions for HAP and binds well to the adsorption sites formed by dislocations or defects of Ca^{2+} on HAP. The high affinity of Ca^{2+} in competition with many kinds of organic cations means that Ca^{2+} can be used as an eluting agent for hydroxyapatite columns.[21] As to competitive adsorption between Ca^{2+} and surfactant cation, the adsorbed amount of Ca^{2+} on HAP is not affected by the addition of cetylpyridinium ion (CP^+) up to 40 mM, whereas the adsorbed amount of CP^+ is greatly decreased by added Ca^{2+}.[22] In the present work, however, adsorption of Ca^{2+} was detected only in the case of high $[CaCl_2]_i$ and low $[DA^+]_f$ (Fig. 3B), where $[Ca^{2+}]_f$ (the equilibrium concentration of Ca^{2+}) was lower than $[CaCl_2]_i$ (=the initial concentration of Ca^{2+}). Moreover, the addition of $CaCl_2$ caused only a slight decrease of the adsorbed amount of DA^+ (X_{DA^+}), as shown in Fig. 1B. These results suggested that dodecylammonium ion has a high affinity for HAP and that the amount of Ca^{2+} released due to ion-exchange exceeded the amount of adsorption of Ca^{2+} on HAP in the case of low $[CaCl_2]_i$ and high $[DA^+]_f$. Thus, the amount of adsorption of Ca^{2+} is apparently negative in this region. Misra *et al.* concluded from the results of electrophoretic mobility measurements that DA^+ forms hemimicelles at the surface of HAP.[23] They also conjectured that the high surface affinity of DA^+ is due to co-adsorption of ammonium ions and amine molecules formed by hydrolysis. However, further discussion is not appropriate at present.

The values of cmc for the systems containing $CaCl_2$ were also determined as a minimum point of $[Pi]_f$ in Fig. 4B (10.1, 9.9 and 8.3 mM for $[CaCl_2]_i=0$, 2.57 and 10.3 mM, respectively). When $[DA^+]_f$ is lower than the cmc, $[Pi]_f$ decreased with increase in $[CaCl_2]_i$. This fact can be explained as follows: adsorbed Ca^{2+} prevents the release of Pi from the surface of HAP through the electrostatic attractive force between them, and excess Ca^{2+} in the solution suppresses the Pi release because the concentrations of Ca^{2+} and Pi in the solution are mutually restricted to keep the solubility product (K_{sp}) for HAP constant (the calculated values for $-\log(Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2$ are 112.19—120.97 and 113.09—119.25 for $[CaCl_2]_i=2.57$ and 10.3 mM).

Even in the region of $[DA^+]_f>$cmc, the Pi release from HAP is depressed. The competitive binding of Cl^- (from $CaCl_2$) and Pi (from HAP) to DA^+ micelles may be

involved in this depression. Of course, the two mechanisms mentioned above, involving adsorbed Ca^{2+} and excess Ca^{2+} in the solution are also available in this region. The latter makes the equilibrium concentration of Pi free from both micelles and HAP low owing to the restriction of K_{sp} for HAP.

Added Ca^{2+} makes the adsorbed amount of DA^+ (X_{DA^+}) decrease by competing for the adsorption sites on HAP. On the other hand, added Cl^- makes X_{DA^+} increase by reducing the electrostatic repulsion among dodecylammonium ions adsorbed on the surface of HAP by virtue of counterion binding and/or increasing the ionic strength. The complicated changes of X_{DA^+} as shown in Fig. 1B can be attributed to these opposite effects of Ca^{2+} and Cl^-

In summary, the amounts of Ca^{2+} and Pi released from HAP depended on the equilibrium concentration of DA^+, the adsorbed amounts of DA^+ and Cl^-, and the concentration of added $CaCl_2$. Furthermore, the concentrations of Ca^{2+} and Pi in the solution were mutually limited so as to keep K_{sp} for HAP constant. When the present results (the surface active ion used is dodecylammonium cation) are compared with those of the previous studies (the surface active ion used was dodecyl sulfate anion),[3,4] it was found that the tendencies for Ca^{2+} and Pi release from HAP in the present work correspond to those of Pi and Ca^{2+} in the previous experiments. That is, the behavior of Pi changed place with that of Ca^{2+}, because the sign of the electric charge of the surface active ion was reversed between the present study and the previous studies.

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A Convenient Synthesis of 1*H*-Pyrrolo[3,2-*c*]quinoline-6,9-diones and 11*H*-Indolo[3,2-*c*]quinoline-1,4-diones Derivatives

PHILIPPE HELISSEY,[a] HÉLÈNE PARROT-LOPEZ,[a] JEAN RENAULT*,[a] and SUZANNE CROS[b]

Laboratoire de Recherche sur les Hétérocycles Azotés, Département de Chimie Organique, Faculté des Sciences Pharmaceutiques et Biologiques de l'Université René Descartes,[a] 75270 Paris Cedex 06, France and Laboratoire de Pharmacologie et de Toxicologie Fondamentales du C.N.R.S.,[b] Toulouse, France

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8-Methoxy-2,3,4-trimethyl-1*H*-pyrrolo[3,2-*c*]quinoline-6,9-dione (**28**) and 2-methoxy-6-methyl-7,8,9,10-tetrahydro-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**31**) can be obtained from 4-hydrazino-6-methoxy-2-methyl-5-quinolinamine (**21**). The synthetic method consists in creating the pyrrole or indole nucleus by applying Fischer's indole synthesis to the hydrazones (**22** and **24**) to give the amines (**23** and **25**, respectively). The catalytic dehydrogenation of **25** leads to the quinolinamine (**26**). The oxidation of the amines (**23**, **25** and **26**) by Fremy's reagent (potassium nitrosodisulfonate) gives the quinones (**28**, **31** and **35**). The nucleophilic substitution of the methoxy group by aziridine leads to **29**, **33** and **36**, which have moderate cytotoxic activity on L 1210 cells.

Keywords——cytotoxicity; Fischer's indole synthesis; Fremy's salt; heterocyclic quinone; 11*H*-indolo[3,2-*c*]quinoline-1,4-dione; 1*H*-pyrrolo[3,2-*c*]quinoline-6,9-dione

A heterocyclic quinone system is present in various antitumor molecules such as streptonigrin[1] (**1**) and it plays important roles in biological systems. The pyrrole nucleus is also found in some antitumor drugs: mitomycin,[2] netropsin[3] and distamycin.[3] An 11*H*-indolo[3,2-*c*]quinoline was found to possess antitumor activity.[4]

Thus, it seemed interesting to study the synthesis of 8-methoxy-1*H*-pyrrolo[3,2-*c*]-quinoline-6,9-diones (**28**, **31**) and 2-methoxy-6-methyl-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**35**), *i.e.*, those quinones derived from a heterocycle obtained by condensation of the quinoline nucleus with the pyrrole or indole nucleus and, like streptonigrin, possessing a methoxy group in the *p*-position relative to the nitrogen of the quinoline nucleus. The synthesis of derivatives of these quinones resulting from nucleophilic substitution of the methoxy group is also described. The cytotoxicity of these heterocycles was briefly examined on L 1210 cells.

Synthesis

The Graebe–Ullman method is the one most commonly used for the synthesis of 11*H*-indolo[3,2-*c*]quinoline. This consists of creating the pyrrole nucleus from 1-(4-quinolyl)-benzotriazole.[5]

However, it was thought to be of greater interest to carry out Fischer's thermal cyclization of hydrazones derived from 4-hydrazinoquinoline. This method provides an easy access to the pyrrolo[3,2-*c*]quinolines[6] and we have extended it to the synthesis of 11*H*-indolo[3,2-*c*]quinolines. The fragility of the *o*-methoxy-*p*-quinone function makes it necessary to introduce this function at the end of the reaction by using a very mild process. To achieve this, two types of compound were chosen (Chart 1): a *p*-dibenzyloxy-*o*-methoxyaryl com-

pound (**2**), which can be selectively hydrogenated to the *o*-methoxy-*p*-diphenol (**3**) and hence oxidized to the quinone (**4**), or an *o*-methoxyarylamine (**15**), which can be oxidized in neutral medium at 20 °C with potassium nitrosodisulfonate (Fremy's reaction)[7] into the *p*-quinone (**4**).

Chart 1

Chart 2

Attempted Preparation of 1*H*-Pyrrolo[3,2-*c*]quinoline-6,9-diones (28, 31) and 11*H*-Indolo[3,2-*c*]quinoline-1,4-dione (35) from a Type 2 Derivative

The attempted synthesis of the 4-quinolone (**11**) required for the preparation of the hydrazine (**12**), in turn required for use in the preparation of the corresponding quinone by reactions analogous to those shown in Charts 3 and 4, was unsuccessful.

The methoxyhydroquinone[8] (**6**) was treated with benzyl chloride in dimethyl sulfoxide[9] and sodium hydroxide to give the triether (**7**) (71%) (Chart 2). Nitration of **7** gave only the nitro derivative (**8**) (93%), the structure of which has been established by means of proton nuclear magnetic resonance (^{1}H-NMR) studies. Reduction with aluminum amalgam[10] gave the benzamine (**9**) (71%). Condensation of **9** with ethyl acetoacetate, under acid catalysis, gave the crotonate (**10**) (91%), identified from the ^{1}H-NMR spectrum (the methyl signal of the CO_2Et at δ 1.2) and infrared (IR) spectrum [bands at 1660 cm^{-1} (C=O) and 3270 cm^{-1} (NH)] (Table I). This crotonate (**10**) is unstable and cannot be cyclized thermally in diphenyl ether to form the 4-quinolone (**11**) (Conrad–Limpach reaction).[11]

Despite the risk of debenzylation, attempts were made to cyclize **10** by using polyphosphoric acid or phosphorus oxychloride, but without success.

Syntheses of 1*H*-Pyrrolo[3,2-*c*]quinoline-6,9-diones (28, 31) and 11*H*-Indolo[3,2-*c*]quinoline-1,4-dione (35) from a Type 5 Derivative

It has already been shown[12] that 7-methoxy-1*H*-pyrrolo[3,2-*c*]quinoline is destroyed by nitration. In addition, 3-methoxy-11*H*-indolo[3,2-*c*]quinoline yields the nitro derivative at C-8, *i.e.*, in the *p*-position relative to the nitrogen of the indole nucleus, and not in the *o*-position

13 → (KNO_3, H_2SO_4) → 14 → ($POCl_3$) → 16; 16 ⇸ 15; 16 ⇸ 17; 15 → (H_2/Ni) → 18; 16 → (MeONa) → 19 → (H_2/Ni) → 20 → ($N_2H_4 \cdot H_2O$) → 21

Chart 3

Table I. Physicochemical Data for the Products

Compd.	mp (°C)	Yield (%)	IR (cm^{-1})[c]		^{1}H-NMR δ (J=Hz)	Formula	Analysis (%) Calcd (Found)		
							C	H	N
7[a]	76—77	71	1120	(OCH_3)	3.75 (3H, s, OCH_3), 4.85 (2H, s, OCH_2 in 1), 4.95 (2H, s, OCH_2 in 4), 6.3 (1H, dd, $J_{5-6}\simeq9$, H_5), 6.5 (1H, d, $J_{3-5}\simeq2$, H_3), 6.65 (1H, d, H_6), 7.25 (10H, m, Ph-H)	$C_{21}H_{20}O_3$	78.75 (78.43	6.25 6.47)	
8[a]	132—133	93	1530, 1260 1070	(NO_2) (OCH_3)	3.80 (3H, s, OCH_3), 5.0 (2H, s, OCH_2 in 1), 5.1 (2H, s, OCH_2 in 4), 6.5 (1H, s, H_3), 7.3 (10H, m, Ph-H), 7.5 (1H, s, H_6)	$C_{21}H_{19}NO_5$	69.04 (69.14	5.20 5.28	3.84 4.01)
9[a]	88	71	3450, 3370 1120	(NH_2) (OCH_3)	3.4 (2H, s, NH_2), 3.65 (3H, s, OCH_3), 4.9 and 4.95 (2H, s, OCH_2), 6.3 and 6.45 (1H, s, H_3 or H_6), 7.25 (10H, m, Ph-H)	$C_{21}H_{21}NO_3$	Not isolated in a pure state		
10[a]	$\simeq$90	91	3270 1660 1060	(NH) (C=O) (OCH_3)	1.2 (3H, t, CH_3), 1.6 (3H, s, CH_3), 3.75 (3H, s, OCH_3), 4.05 (2H, q, CH_2), 4.55 (H, s, vinyl-H), 4.95 and 5.0 (2H, s, OCH_2), 6.5 and 6.55 (1H, s, H_3 or H_6), 7.25 (10H, m, Ph-H), 9.9 (1H, s, NH)	$C_{27}H_{29}NO_5$	Not isolated in a pure state		
14[b]	>335	86	2750 1595 1520, 1370 1065	(NH) (C=O) (NO_2) (OCH_3)	2.25 (3H, s, CH_3), 3.8 (3H, s, OCH_3), 5.8 (1H, s, H_3), 7.6 (2H, s, H_7 and H_8), 11.8 (1H, s, NH)	$C_{11}H_{10}N_2O_4$	56.41 (56.27	4.27 4.27	11.97 12.02)
16[b]	122—123	81	1535, 1375 1085	(NO_2) (OCH_3)	2.6 (3H, s, CH_3), 4.0 (3H, s, OCH_3), 7.75 (1H, s, H_3), 7.9 (1H, d, $J_{7-8}\simeq9$, H_7), 8.2 (1H, d, H_8)	$C_{11}H_9ClN_2O_3$	52.28 (52.43	3.56 3.40	11.09 11.12)
18[b]	125—126	45	3450, 3360 1090	(NH_2) (OCH_3)	2.5 (3H, s, CH_3), 3.8 (3H, s, OCH_3), 5.3 (2H, s, NH_2), 7.1 and 7.15 and 7.35 (1H, d, $J\simeq9$, H_3 or H_7 or H_8), 8.3 (1H, d, H_4)	$C_{11}H_{12}N_2O\cdot H_2O$	64.07 (63.91	6.79 6.76	13.59 13.69)
19[b]	154—155	82	1525, 1365 1060	(NO_2) (OCH_3)	2.55 (3H, s, CH_3), 3.85 (3H, s, OCH_{3-6}), 3.95 (3H, s, OCH_{3-4}), 7.0 (1H, s, H_3), 7.75 (1H, d, $J_{7-8}\simeq9$, H_7), 8.0 (1H, d, H_8)	$C_{12}H_{12}N_2O_4$	58.06 (57.99	4.84 4.73	11.29 11.26)
20[b]	148—149	96	3490, 3390 1110, 1080	(NH_2) (OCH_3)	2.45 (3H, s, CH_3), 3.8 (3H, s, OCH_{3-6}), 3.9 (3H, s, OCH_{3-4}), 5.55 (2H, s, NH_2), 6.55 (1H, s, H_3), 7.0 and 7.25 (1H, d, $J_{7-8}\simeq9$, H_7 or H_8)	$C_{12}H_{14}N_2O_2$	66.05 (65.66	6.42 6.62	12.84 13.10)
21[b]	212—213	75	3400, 3310 3170 1100	(NH_2) (NH) (OCH_3)	1.95 (3H, s, CH_3), 3.65 (3H, s, OCH_3), 4.9 (2H, s, NH_2), 5.4 (1H, s, H_3), 6.05 and 6.55 (1H, d, $J_{7-8}\simeq$ 9, H_7 or H_8), 6.85 (2H, s, NH_2), 9.3 (1H, s, NH)	$C_{11}H_{14}N_4O$	60.55 (60.26	6.42 6.42	25.69 25.85)

23[b]	252—253	60	3395, 3320 3250 1080	(NH_2) (NH) (OCH_3)	2.35 (6H, s, CH_{3-2} and CH_{3-3}), 2.75 (3H, s, CH_{3-4}), 3.85 (3H, s, OCH_3), 4.85 (2H, s, NH_2), 7.15 and 7.20 (1H, d, $J_{6-7}\simeq 9$, H_6 or H_7), 10.9 (1H, s, NH)	$C_{15}H_{17}N_3O\cdot H_2O$	65.93 (66.01	6.96 6.90	15.39 15.32)
25[b]	251—252	85	3380, 3310 3230 1075	(NH_2) (NH) (OCH_3)	1.8 (4H, m, $2CH_2$), 2.7 (3H, s, CH_3), 2.75 (2H, m, CH_2), 2.9 (2H, s, CH_2), 3.85 (3H, s, OCH_3), 4.9 (2H, s, NH_2), 7.15 and 7.2 (1H, d, $J_{3-4}\simeq 9$, H_3 or H_4), 11.0 (1H, s, NH)	$C_{17}H_{19}N_3O\cdot H_2O$	68.23 (67.88	7.02 7.01	14.05 14.43)
26[b]	269—270	47	3400, 3100	(NH_2)	2.95 (3H, s, CH_3), 3.85 (3H, s, OCH_3), 7.15 (2H, s, NH_2), 7.2 (1H, t, $J_{8-9}\simeq 8$, H_8), 7.3 (3H, m, H_3 and H_4 and H_9), 7.7 (1H, d, $J_{10-9}\simeq 8$, H_{10}), 8.05 (1H, d, $J_{7-8}\simeq 8$, H_7), 12.2 (1H, s, NH)	$C_{17}H_{15}N_3O\cdot H_2O$	69.15 (69.36	5.76 5.73	14.24 14.35)
28[b]	297—298	79	3640, 3350 3180 1670, 1645 1050	(OH) (NH) (C=O) (OCH_3)	2.3 and 2.35 (3H, s, CH_{3-2} and CH_{3-3}), 2.8 (3H, s, CH_{3-4}), 3.8 (3H, s, OCH_3), 6.15 (1H, s, H_7), 11.75 (1H, s, NH)	$C_{15}H_{14}N_2O_3\cdot 1.5MeOH$	62.26 (62.59	6.29 5.96	8.81 9.07)
29[b]	218—219	38	3360 1650	(NH) (C=O)	2.2 (4H, m, N–CH_2), 2.30 and 2.35 (3H, s, CH_{3-2} and CH_{3-3}), 2.8 (3H, s, CH_{3-4}), 6.15 (1H, s, H_7), 11.75 (1H, s, NH)	$C_{16}H_{15}N_3O\cdot 0.5H_2O$	67.25 (66.91	5.43 5.32	14.71 14.55)
31[b]	256—257	70	3330 1650 1070	(NH) (C=O) (OCH_3)	1.75 (4H, m, $2CH_2$), 2.75 (5H, m, CH_3 and CH_2), 2.85 (2H, m, CH_2), 3.8 (3H, s, OCH_3), 6.15 (1H, s, H_3), 11.75 (1H, s, NH)	$C_{17}H_{16}N_2O_3$	68.92 (69.20	5.41 5.51	9.46 9.16)
32[b]	262—263	45	3200 1650	(NH) (C=O)	1.6 (6H, m, $3CH_2$), 1.75 (4H, m, $2CH_2$), 2.7 (5H, m, CH_3 and CH_2), 2.85 (2H, m, CH_2), 3.4 (4H, m, $2CH_2$), 5.8 (1H, s, H_3), 11.65 (1H, s, NH)	$C_{21}H_{23}N_3O_2$	72.21 (72.36	6.59 6.66	12.03 12.26)
33[b]	178—179	55	3340 1645	(NH) (C=O)	1.75 (4H, m, $2CH_2$), 2.2 (4H, m, N–CH_2), 2.7 (5H, m, CH_3 and CH_2), 2.85 (2H, m, CH_2), 6.15 (1H, s, H_3), 11.75 (1H, s, NH)	$C_{18}H_{17}N_3O_2\cdot 1.25H_2O$	65.55 (65.69	5.91 5.84	12.74 12.50)
35[b]	310—311	19	3390 1650 1070	(NH) (C=O) (OCH_3)	3.0 (3H, s, CH_3), 3.85 (3H, s, OCH_3), 6.25 (1H, s, H_3), 7.3 (1H, t, $J_{8-9}\simeq 8$, H_8), 7.5 (1H, t, $J_{9-10}\simeq 8$, H_9), 7.8 (1H, d, H_{10}), 8.15 (1H, d, $J_{7-8}\simeq 8$, H_7), 12.35 (1H, s, NH)	$C_{17}H_{12}N_2O_3$	67.77 (67.48	4.32 4.34	9.30 9.54)
36[b]	208—209	45	3360 1650	(NH) (C=O)	2.25 (4H, m, N–CH_2), 3.0 (3H, s, CH_3), 6.25 (1H, s, H_3), 7.3 (1H, t, $J_{8-9}\simeq 8$, H_8), 7.5 (1H, t, $J_{9-10}\simeq 8$, H_9), 7.75 (1H, d, H_{10}), 8.15 (1H, d, $J_{7-8}\simeq 8$, H_7), 12.4 (1H, s, NH)	$C_{18}H_{13}N_3O_2\cdot 0.5H_2O$	69.23 (69.43	4.49 4.22	13.46 13.11)

a) ^{1}H-NMR spectra were measured in $CDCl_3$. *b)* ^{1}H-NMR spectra were measured in DMSO-d_6. *c)* IR spectra were measured by the KBr disc method.

with respect to the methoxy group.[12] Consequently, an attempt was made to obtain the desired primary amines (**23**, **25** and **26**) directly from 4-hydrazino-6-methoxy-2-methyl-5-quinolinamine (**21**) (Chart 3).

This synthesis was initiated from 4-chloro-6-methoxy-2-methyl-5-nitroquinoline (**16**). This compound, which was described by Heindel *et al.*,[13] can be obtained in a yield of only 58% from the nitration of the corresponding 4-chloroquinoline, which can itself be prepared by chlorination of the 6-methoxy-2-methyl-4(1*H*)-quinolinone (**13**) (the yield was not specified), according to Basu and Chaudran.[14] We improved the method and brought the total yield to 69% from the 4-quinolinone (**13**) by reversing the order of the reactions (nitration of **13** followed by chlorination of **14**).

The sensitivity to nucleophilic substitutions of the methoxy group did not allow us to obtain the nitrated hydrazine (**17**), which is liable to form the aminohydrazine (**21**) by reduction. Thus, we tried to obtain it from 4-chloro-6-methoxy-2-methyl-5-quinolinamine (**15**) which can be prepared by the catalytic reduction of the nitro group of **16**. Catalytic reduction in the presence of Raney nickel in various solvents, chemical reduction with sodium borohydride in the presence of tin(II) chloride according to Satoh *et al.*[15] or reduction with iron in acetic medium according to Price *et al.*,[16] who selectively reduced the nitro group of 4-chloro-6-methoxy-8-nitroquinoline, led to complete hydrogenolysis of the C–Cl bond with the formation of 6-methoxy-2-methyl-5-quinolinamine (**18**).

We were able to solve this problem by substituting a methoxy group in place of the chlorine atom of **16** by the use of sodium methoxide. The nitro compound (**19**) thus obtained in a yield of 82% can then be easily reduced to an amino derivative (**20**).

Though Buchmann and Hamilton[17] showed that morpholine reacts with 2-chloro-4-ethoxyquinoline or 4-chloro-2-ethoxyquinoline without affecting the ethoxy group, we were hoping that the methoxy group at C_4 of **20** would be made labile by the $-M$ effect of nitrogen, as noted in other hexagonal heterocyclic series containing methoxy groups located *ortho* or *para* to intracyclic nitrogen, *e.g.*, in 3-methoxy-1,2,4-triazine[18] and 2-chloro-4-ethoxyquinazoline.[19] The methoxy derivative (**20**) readily gave the hydrazine (**21**) in 75% yield when treated with hydrazine hydrate.

The hydrazones (**22** and **24**) can be synthesized from the reaction of hydrazine (**21**) with

Chart 4

butanone or cyclohexanone in an alcoholic medium (Chart 4). According to Fischer's indole synthesis, the amines (**23** and **25**) can be obtained through the thermal cyclization of **22** and **24**.

The aromatization of the amine (**25**) in the presence of palladium on carbon facilitates access to the 2-methoxy-6-methyl-11*H*-indolo[3,2-*c*]quinolin-1-amine (**26**).

The elemental analyses and IR and ^{1}H-NMR spectral data were consistent with the assigned structures (Table I).

Quinones were prepared as indicated in Chart 5 by oxidation of the corresponding amines (**23**, **25** and **26**) with potassium nitrosodisulfonate. The oxidation reaction leads to a quinonimine intermediate, which was not isolated in a pure state. For example, the oxidation of **23** exclusively gives the imine (**27**), characterized by ^{1}H-NMR spectroscopy. However, oxidation of **25** and **26** gave a 1 : 1 mixture of quinonimines (**30** and **34**) and quinones (**31** and **35**) (^{1}H-NMR quantitative analysis). The quinonimine (**30**) was purified through preparative chromatography and identified by ^{1}H-NMR spectroscopy, but could not be completely separated from the quinone (**31**). These imines are rapidly hydrolyzed into quinones by acid

Chart 5

TABLE II. Cytotoxic Effects of 8-Substituted 1*H*-Pyrrolo[3,2-*c*]quinoline-6,9-diones (**28**, **29**) and 2-Substituted 11*H*-Indolo[3,2-*c*]quinoline-1,4-diones (**31** to **36**) on Growth of L 1210 Cells *in Vitro*

Compound No.	IC_{50}[a] ng/ml	IC_{50}[a] 10^{-6} M
28	687	2.16
29	147	0.514
31	1336	4.51
32	3553	10.2
33	476	1.44
35	>1000	>33.2
36	238	0.762

a) Concentration that reduces the L 1210 cells growth *in vitro* by 50% after 48 h as compared to the control.

treatment.

The methoxy group *ortho* to the quinone is labile to nucleophilic reagents. It seemed to us that aziridine derivatives would be interesting because of their bioalkylating properties.[20] The quinones (**29**, **33** and **36**) were obtained from the corresponding quinones (**28**, **31** and **35**) in the presence of an excess of amine. Thin-layer chromatography (TLC) showed the presence of a major compound, despite tar formation.

Pharmacology

***In Vitro* Cytotoxicity on L 1210 Leukemia Cells**——Cytotoxicity towards L 1210 leukemia cells was determined. Dose-effect relationships of the various compounds tested were determined from the regression line of percent cell growth inhibition plotted as a function of the logarithm of the dose. From these curves, the dose of drug which reduces the cell growth by 50% after 48 h as compared to the control was estimated. As shown in Table II, the compounds tested are cytotoxic to L 1210 cells.

Discussion

Replacement of the methyl groups at the C-2 and C-3 positions of 1*H*-pyrrolo[3,2-*c*]quinoline-6,9-diones (**28** and **29**) with a tetramethylene chain **31** and **33** had little effect on the cytotoxicity. Replacing the methyl groups of **28** by a divinyl chain **35** is extremely unfavorable, though in the aziridine (**29**), this modification is without effect. Substituting the methoxy group of **31** for piperidine (**32**) lowered the cytotoxicity. On the other hand, substituting a methoxy group for the 1-aziridinyl group leads to the quinones (**29**, **33** and **36**), which are more cytotoxic. The increase of cytotoxicity is most probably due to the alkylating action of this 1-aziridinyl group.

Experimental

All melting points were determined on a Maquenne apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer 157G spectrometer. NMR spectra were measured with a Bruker 270 MHz spectrometer with trimethylsilane $(Me_3Si)_2$ as an internal reference. TLC was carried out on Merck GF 254 silica gel plates.

Chemistry

1,4-Dibenzyloxy-2-methoxybenzene (7)——Benzyl chloride (23 ml, 200 mmol) was added to a solution of 7 g (50 mmol) of methoxyhydroquinol (**6**)[8] and 22.5 g (400 mmol) of potassium hydroxide in 50 ml of dimethyl sulfoxide (DMSO) at 20 °C. The mixture was stirred for 3 h, then H_2O (500 ml) was added and the whole was extracted with CH_2Cl_2. The solvent layer was washed with H_2O, dried, treated with charcoal and evaporated under reduced

pressure. The product was recrystallized from EtOH to give **7** (11.36 g).

1,4-Dibenzyloxy-2-methoxy-5-nitrobenzene (8)——Nitric acid (1.8 ml, 40 mmol, d=1.42) was added to a suspension of 3.2 g (10 mmol) of **7** in 25 ml of acetic acid at 20 °C. The mixture was stirred for 1 h, then H_2O (200 ml) was added. The solid that separated was filtered off, washed with H_2O and recrystallized from EtOH to give **8** (3.39 g).

2,5-Dibenzyloxy-4-methoxy-benzenamine (9)——A solution of 1.82 g (5 mmol) of **8** in 10 ml of tetrahydrofuran (THF) was added slowly to a suspension of 2 g aluminum amalgam shot in 10 ml EtOH–THF (1 : 1). The mixture was refluxed under N_2 for 3 h, then allowed to cool. The solid was filtered off, and washed with THF. The solvent was distilled off. The residue was unstable and could not be isolated in a pure state.

Ethyl 3-(2,5-Dibenzyloxy-4-methoxyphenylamino)crotonate (10)——Crude **9** (335 mg, 1 mmol) in 3 ml of benzene, 144 mg (1.1 mmol) of ethyl acetoacetate and a drop of 2 M ethanolic HCl solution were heated at 80 °C under N_2 for 3.5 h. The solvent was distilled off under reduced pressure and the residue was washed with petroleum ether. The precipitate was filtered but was not further purified.

6-Methoxy-2-methyl-5-nitro-4(1*H*)-quinolinone (14)——Potassium nitrate (5.3 g, 52.5 mmol) was added to a solution of 9.45 g (50 mmol) of 6-methoxy-2-methyl-4(1*H*)-quinolinone (**13**)[11] in 100 ml of sulfuric acid (d 1.84) at 0 °C. The mixture was stirred at 0 °C for 4 h before being poured on ice; ammonia (d 0.9) was added while cooling until pH 7 was reached. The solid was separated by filtration washed with H_2O, dried and recrystallized from EtOH to give **14** (10.06 g).

4-Chloro-6-methoxy-2-methyl-5-nitroquinoline (16)——Compound **14** (10.5 g, 45 mmol) was added to 55 ml of $POCl_3$ at 20 °C. The mixture was stirred at 105 °C for 4 h. The $POCl_3$ was evaporated off *in vacuo* and the residue was poured into a mixture of ice-ammonia (d 0.9) and extracted with CH_2Cl_2. The solvent layer was washed with water, dried, treated with charcoal and evaporated under reduced pressure. The product was recrystallized from EtOH–H_2O to give **16** (9.20 g).

6-Methoxy-2-methyl-5-quinolinamine (18)——A solution of 0.252 g (1 mmol) of **16** in 10 ml of MeOH was hydrogenated in the presence of 0.25 g of Raney nickel until the gas uptake ceased (4 mmol). After filtration to remove the catalyst under N_2, the mixture was treated with charcoal and evaporated. The residue was dissolved in 200 ml of boiling water, 1 M NaOH solution was added to give pH 8, and the mixture was extracted with CH_2Cl_2. The solvent layer was washed with water, dried, treated with charcoal and evaporated. This product was recrystallized from EtOH–H_2O to give **18** (0.085 g).

4,6-Dimethoxy-2-methyl-5-nitroquinoline (19)——A solution of 18.9 g (75 mmol) of **16** and 16.2 g (300 mmol) of sodium methoxide in 400 ml of dry MeOH was stirred for 24 h under reflux. The solvent was evaporated off under reduced pressure. The residue was dissolved in 100 ml of H_2O, HCl solution (d 1.19) was added to give pH 7, and the solution was extracted with $CHCl_3$. The combined solvent layers were washed with water, dried, treated with charcoal and evaporated to dryness. The residue was recrystallized from EtOH–H_2O to give **19** (15.25 g).

4,6-Dimethoxy-2-methyl-5-quinolinamine (20)——A solution of 4.96 g (20 mmol) of **19** in 200 ml of a 1 : 1 mixture of MeOH and dioxane was hydrogenated over 5 g of Raney nickel until the gas uptake ceased (68 mmol). After filtration to remove the catalyst under N_2, the mixture was treated with charcoal and evaporated to give 4.18 g of **20**. Recrystallized from EtOH–H_2O.

4-Hydrazino-6-methoxy-2-methyl-5-quinolinamine (21)——A suspension of 4.14 g (19 mmol) of **20** in 100 ml of hydrazine hydrate was heated at 120 °C under N_2 for 5 h. The precipitate was filtered off, washed with H_2O and dried to give 3.10 g of pure product.

8-Methoxy-2,3,4-trimethyl-1*H*-pyrrolo[3,2-c]quinolin-9-amine (23)——Compound **21** (1.09 g, 5 mmol) in 10 ml of EtOH and 1.08 g (15 mmol) of butanone were heated under reflux for 3 h. The solvent was evaporated off under reduced pressure, and the residue was washed with petroleum ether to give 1.08 g of the hydrazone (**22**) (yield, 80%). The hydrazone (1.36 g, 5 mmol), without purification, was taken up in 10 ml of diethylene glycol and heated at 250 °C under N_2 for 45 min. The reaction mixture was cooled and diluted with 35 ml of H_2O. The precipitate was filtered off, washed with H_2O and then crystallized from aqueous ethanol to give 0.76 g of **23**.

2-Methoxy-6-methyl-7,8,9,10-tetrahydro-11*H*-indolo[3,2-*c*]quinolin-1-amine (25)——Compound **21** (1.09 g, 5 mmol) in 10 ml of EtOH and 0.54 g (5.5 mmol) of cyclohexanone were heated under reflux for 2 h. The solvent was evaporated off under reduced pressure, and the residue was washed with petroleum ether to give 1.49 g of the hydrazone (**24**) (yield, 80%). The hydrazone (1.49 g, 5 mmol), without purification, was treated in the same manner as **22**. This product was recrystallized from aqueous ethanol to give 1.27 g of **25**.

2-Methoxy-6-methyl-11*H*-indolo[3,2-*c*]quinolin-1-amine (26)——A suspension of 1.82 g (6.5 mmol) of **25** and 2 g of 10% palladium on activated charcoal in 30 ml of decalin was refluxed under N_2 for 10 h, then allowed to cool. The solid was filtered off, extracted with $CHCl_3$–MeOH (1 : 1), dried, treated with charcoal and evaporated to give 0.91 g of **26**. Recrystallized from EtOH–H_2O.

General Procedure for Synthesis of 28, 31 and 35——A solution of 1.36 g (10 mmol) of monobasic potassium phosphate in x ml of H_2O was added to 2 mmol of amino compound (**23**, **25** or **26**) in y ml of acetone–MeOH (1 : 1). Potassium nitrosodisulfonate (2.15 g, 8 mmol) was added over 10 min. The mixture was stirred for m h, then 2 M HCl solution (5 ml) and 40 ml of MeOH were added and the whole was stirred for an additional n h. $NaHCO_3$ saturated solution was added to give pH 7. The organic solvent was removed under reduced pressure, and the residue was

extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried and evaporated to dryness to give a pyrroloquinoline-6,9-dione. The amine (**26**) gave a precipitate of indoloquinoline-1,4-dione. This precipitate was filtered off, and extracted with $CHCl_3$–MeOH (1 : 1). The extract was treated with charcoal and evaporated.

8-Methoxy-2,3,4-trimethyl-1*H*-pyrrolo[3,2-*c*]quinoline-6,9-dione (**28**): $x=30$, $y=60$, $m=n=3$. The product was recrystallized from MeOH to give 0.502 g of **28**.

2-Methoxy-6-methyl-7,8,9,10-tetrahydro-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**31**): $x=30$, $y=60$, $m=2$, $n=3$. The product was recrystallized from CH_3CN to give 0.414 g of **31**.

2-Methoxy-6-methyl-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**35**): $x=40$, $y=70$, $m=24$, $n=5$. The product was recrystallized from MeOH to give 0.111 g of **35**.

Remarks——The imines (**27**, **30** and **34**) were prepared in the same manner as **28**, **31** and **35**. HCl solution was not added. These compounds were identified by ^{1}H-NMR analysis and not isolated in a pure state.

9-Imino-8-methoxy-6-oxo-2,3,4-trimethyl-6,9-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline (**27**): ^{1}H-NMR (DMSO-d_6) δ: 2.3 and 2.35 (3H, s, Me-2 and Me-3), 2.8 (3H, s, Me-4), 3.9 (3H, s, CH_3O), 5.95 (1H, s, H_7), 11.2 and 11.45 (1H, s, NH).

1-Imino-2-methoxy-6-methyl-4-oxo-1,4,7,8,9,10-hexahydro-11*H*-indolo[3,2-*c*]quinoline (**30**): The precipitate was purified on preparative alumina plate (solvent: $CHCl_3$). ^{1}H-NMR (DMSO-d_6) δ: 1.75 (4H, m, $2CH_2$), 2.75 (5H, m, CH_2 and CH_3), 2.85 (2H, m, CH_2), 3.9 (3H, s, CH_3O), 5.95 (1H, s, H_3), 11.3 and 11.45 (1H, s, NH).

1-Imino-2-methoxy-6-methyl-4-oxo-1,4-dihydro-11*H*-indolo[3,2-*c*]quinoline (**34**): ^{1}H-NMR (DMSO-d_6) δ: 3.0 (3H, s, CH_3), 3.9 (3H, s, CH_3O), 6.05 (1H, s, H_3), 7.3 (1H, t, $J_{8-9}\simeq 8$ Hz, H_8), 7.5 (1H, t, $J_{9-10}\simeq 8$ Hz, H_9), 7.9 (1H, d, H_{10}), 8.15 (1H, d, $J_{7-8}\simeq 8$ Hz, H_7), 11.65 and 12.0 (1H, s, NH).

General Procedure for Synthesis of 29, 33 and 36——Aziridine (2 ml, 40 mol) was added to a suspension of 0.5 mmol of the quinone (**28**, **31** or **35**) in 5 ml of dry MeOH. The mixture was stirred at 20 °C for *n* h. The solid that separated was filtered off, and washed with petroleum ether.

8-(1-Aziridinyl)-2,3,4-trimethyl-1*H*-pyrrolo[3,2-*c*]quinoline-6,9-dione (**29**): $n=4$. The product was recrystallized from MeOH to give 0.052 g of **29**.

2-(1-Aziridinyl)-6-methyl-7,8,9,10-tetrahydro-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**33**): $n=2$. The product was recrystallized from MeOH to give 0.091 g of **33**.

2-(1-Aziridinyl)-6-methyl-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (**36**): $n=12$. The product was recrystallized from MeOH to give 0.070 g of **36**.

6-Methyl-2-piperidino-7,8,9,10-tetrahydro-11*H*-indolo[3,2-*c*]quinoline-1,4-dione (32)——Piperidine (5 ml, 50 mmol) was added to a suspension of 0.148 g (0.5 mmol) of **31** in 5 ml of dry MeOH. The mixture was stirred at 20 °C for 1 h. The solvent was evaporated off under reduced pressure. The product was recrystallized from CH_3CN to give 0.078 g of **32**.

Pharmacology

Growth Inhibition of L 1210 Cells in Culture——The experimental protocol has been reported.[22]

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Synthesis and Properties of Deoxyoligonucleotides Containing Putrescinylthymine (Nucleosides and Nucleotides. LXXVI)[1]

TADAYUKI TAKEDA,[a] KAZUYOSHI IKEDA,[b] YOSHIHISA MIZUNO,[a] and TOHRU UEDA*,[a]

Faculty of Pharmaceutical Sciences[a] and Center for Instrumental Analysis,[b] Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan

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Putrescinylthymidine was prepared by the reduction of the Schiff base formed from a 2′-deoxy-5-formyluridine derivative and *N*-phthaloylputrescine, followed by deprotection. The following deoxyoligonucleotides containing putrescinylthymine (T^P) were synthesized; dodecathymidylic acids containing two to four T^P residues, self-complementary decanucleotides (AAGAATTCTT) and dodecanucleotides (AGATAGCTATCT) in which T residues were partly replaced by T^P, and related oligomers. Oligonucleotides containing T^P were resistant to nuclease S1 digestion and were poor substrates to venom phosphodiesterase. The thermal stability (T_m) of the duplex structure of oligomers containing T^P was not enhanced in spite of the expected electrostatic binding between the putrescinyl and phosphoryl residues, and was rather sequence-dependent.

Keywords——deoxyoligonucleotide; putrescinylthymine; putrescinylthymidine; melting temperature (T_m); duplex stability; nuclease S1; venom phosphodiesterase

It is well known that bacteriophage deoxyribonucleic acids (DNAs) contain various modified pyrimidine bases such as 5-hydroxymethylcytosine,[2] 5-methylcytosine,[3] 5-hydroxymethyluracil,[4] 5-(4,5-dihydroxypentyl)uracil,[5] α-glutaminylthymine,[6] and putrescinylthymine (5-(4-aminobutylamino)methyluracil, T^P, the base moiety of **1**).[7] Among them, the hypermodified bases[5-7] have attracted much interest in relation to their functions in these DNAs. For example, phage ΦW-14 DNA contains up to 50% T^P in place of thymine.[7,8] This modification results in a higher melting temperature (T_m, 99.3 °C) than expected from the GC content (90.3 °C), and lower buoyant density (1.666) than that expected (1.716).[7,9] The enhanced thermal stability of this DNA has been explained in terms of the reduction of the anionic electrostatic repulsion between phosphoryl moieties by the presence of the cationic putrescinyl groups.[9] A recent report suggested a function of this basic moiety in the efficient packaging of the DNA in the virus particle.[10]

Since the T^P moieties in the DNA are not modified any further, unlike other modified bases such as 5-hydroxymethylcytosine, a study of oligonucleotides containing T^P should be useful for direct estimation of the function of this base moiety in the viral DNA. This paper describes the synthesis of putrescinylthymidine and deoxyoligonucleotides of defined sequence containing T^P in place of thymine, and the properties of these modified oligomers. A preliminary account of a part of this work has appeared.[11]

Synthesis of Putrescinylthymidine

Initial attempts at obtaining putrescinylthymidine (**1**) by substitution of 5-acetoxymethyl-3′,5′-di-*O*-benzoyl-2′-deoxyuridine or 3′,5′-di-*O*-acetyl-5-bromomethyl-2′-deoxyuridine with N^1-phthaloylputrescine resulted in a low yield of **1**. We have recently found that the 5-aminomethyluridines can be obtained by the Schiff base formation of 5-formyluridine with amines followed by reduction with borohydride.[12] This route seemed to

be useful for the present purpose. 3′,5′-Di-*O*-acetyl-5-formyl-2′-deoxyuridine (**2**), prepared by oxidation of the thymidine with potassium peroxysulfate,[13] was treated with *N*-phthaloylputrescine (**3**) in dioxane at room temperature to give the Schiff base **4**, which was further treated with sodium borohydride at low temperature. After chromatographic purification of the product, 3′,5′-di-*O*-acetyl-*N*-phthaloylputrescinylthymidine (**5**) was obtained in 30% overall yield. Ammonolysis of **5** afforded putrescinylthymidine (**1**), isolated as the mono-oxalate. The structure of **1** was confirmed by the nuclear magnetic resonance

Chart 1

(NMR) spectrum and the result of elemental analysis. As reference compounds, 2′-deoxy-5-(methylaminomethyl)uridine (**6**) and 2′-deoxy-5-(methoxymethyl)uridine (**7**) were prepared from 3′,5′-di-*O*-acetyl-5-bromomethyl-2′-deoxyuridine by methylammonolysis or methanolysis, respectively.

Synthesis of Deoxyoligonucleotides Containing Putrescinylthymine (T^P), 5-(Methylaminomethyl)uracil (T^N), and 5-(Methoxymethyl)uracil (T^O)

Prior to oligonucleotide synthesis, the side-chain amino groups of **1** and **6** were protected with a trifluoroacetyl function by treatment with ethyl trifluoroacetate in the presence of triethylamine[14] in methanol to give *N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (T^{PF}, **8**) and *N*-trifluoroacetyl-5-methylaminomethyl-2′-deoxyuridine (T^{NF}, **9**), respectively. These compounds and **7** were then converted to the 5′-dimethoxytrityl derivatives (**10**, **11**, **12**) by the usual procedure. Compound **10** was further converted to the 3′-*O*-acetyl derivative (**13**), 3′-phosphotriesters (**14** and **15**) and 3′-phosphodiester (**16**) by the usual procedures.[15,16] Compounds **11** and **12** were also converted to the properly protected nucleotides, and these monomer units were used in oligonucleotide synthesis. Dinucleotide monophosphate, T^PT, was prepared by condensation of **16** with 3′-*O*-acetylthymidine by the use of triisopropylbenzenesulfonyl chloride (TPSCl) in the presence of *N*-methylimidazole,[17] followed by deprotection. TT^P was likewise prepared from **13** and the appropriate nucleotide component.

The oligonucleotides TTCTT, TT^PCTT, $TTCT^PT$, AAGAATTCTT, $AAGAATT^PCTT$, $AAGAATTCT^PT$, and $AAGAATT^PCT^PT$ were prepared by the liquid phase phosphotriester method.[17] The results at the stage of the final condensation steps are summarized in Table I. The deprotection was performed by successive treatment with *N*,*N*,*N*′,*N*′-tetramethylguanidinium *p*-nitrobenzaldoximate (TMGNBO),[18] conc. NH_4OH, and 80% AcOH. The oligomers were purified by diethylaminoethyl (DEAE)-cellulose column chromatography or reversed phase column chromatography,[19] and high performance liquid chromatography (HPLC). The dodecanucleotide AGATAGCTATCT and analogs containing T^P in place of T at positions 4, 8, and 10 were prepared by the solid-phase phosphotriester method using aminomethylpolystyrene as the support.[20] Dodecathymidylate (T_{12}) and analogs containing T^P at positions 3 and 9 ($T^P_{3,9}$), 3, 7, and 11 ($T^P_{3,7,11}$), and 2, 5, 8, 11 ($T^P_{2,5,8,11}$) were also prepared. Similarly, $T^P_{3,9}$ and $T^O_{3,9}$ were prepared. The results are summarized in Table II with the yield of each condensation step. The oligomers prepared by this method were purified by a combination of gel-filtration and HPLC to a purity of >99%.

Digestion of Oligonucleotides by Nucleolytic Enzymes

It has been reported that ΦW-14 DNA is resistant to deoxyribonuclease (DNase) I or venom phosphodiesterase (VPDase) digestion[21] and T^P-nucleoside was detectable after nuclease S1 digestion of heat-denatured DNA followed by VPDase and alkaline phosphatase (APase) action.[22] Therefore, T_{12} and analogs containing T^P, T^N or T^O were treated with nuclease S1, VPDase, and nuclease P_1 to test their susceptibility. Nuclease S1 completely cleaved T_{12} at 50 °C overnight, giving 5′-TMP (pdT) and thymidine (dT) in 11 : 1 ratio. Under similar conditions $T^P_{3,9}$, $T^N_{3,9}$, and $T^O_{3,9}$ showed several peaks other than those of pdT and dT on HPLC, suggesting resistance due to modification of the methyl function of thymine residue.

VPDase (with APase) cleaved $T^O_{3,9}$ completely to give dT and dT^O in the expected ratio. $T^N_{3,9}$ showed one peak in addition to those of dT and dT^N. In the case of $T^P_{3,9}$, dT^P was not detected, and instead, two peaks probably due to incomplete hydrolysis were detected. One of them was confirmed to be TT^P by co-chromatography with an authentic sample, and the other peak was assumed to be TTT^P or $TTTT^P$, since its retention time was not identical with that of authentic T^PT. In fact, VPDase digestion of T^PT gave dT and dT^P, while TT^P was completely resistant.

TABLE I. Synthesis of Penta- and Decadeoxyribonucleotides Containing T^{P}

3′-Diester component mg (mmol)	5′-Hydroxy component mg (mmol)	*N*-Methyl-imidazole mg (mmol)	TPSCl mg (mmol)	Reaction time h	Oligomer mg yield (%)	Deprotected oligomer (T_m)
DMTrTTp 250 (0.2)	$C^{an}TT_{ac}$ 222 (0.18)	98 (1.2)	182 (0.6)	2	$DMTrTTC^{an}TT_{ac}$ 230 (75)	TTCTT
$DMTrTT^{PE}p$ 90 (0.06)	$C^{an}TT_{ac}$ 62 (0.05)	33 (0.4)	50 (0.16)	3	$DMTrTT^{PF}C^{an}TT_{ac}$ 90 (65)	$TT^{P}CTT$
$DMTrTT^{PF}p$ 105 (0.07)	$C^{an}T^{PF}T_{ac}$ 62 (0.05)	40 (0.48)	55 (0.18)	1.5	$DMTrTT^{PF}C^{an}T^{PF}T_{ac}$ 114 (65)	$TT^{P}CT^{P}T$
$DMTrTTC^{an}p$ 70 (0.04)	$T^{PF}T_{ac}$ 30 (0.03)	16 (0.26)	36 (0.12)	1.5	$DMTrTTC^{an}T^{PF}T_{ac}$ 77 (78)	$TTCT^{P}T$
$DMTrA^{bz}A^{bz}G^{ib}p$ 128 (0.07)	$A^{bz}A^{bz}p$(CE) 68 (0.06)	33 (0.4)	61 (0.2)	1	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$(CE) 120 (70)	
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 30 (0.01)	$TTC^{an}TT_{ac}$ 18 (0.008)	9 (0.1)	16 (0.05)	2	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}TT_{ac}$ 33 (56)	AAGAATTCTT (22 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 36 (0.012)	$TT^{PF}C^{an}TT_{ac}$ 24 (0.01)	11 (0.13)	20 (0.06)	4	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}TT_{ac}$ 39 (77)	$AAGAATT^{P}CTT$ (16 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 40 (0.015)	$TTC^{an}T^{PF}T_{ac}$ 37 (0.015)	8 (0.1)	15 (0.05)	1	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TTC^{an}T^{PF}T_{ac}$ 42 (68)	$AAGAATTCT^{P}T$ (27 °C)
$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}p$ 40 (0.015)	$TT^{PF}C^{an}T^{PF}T_{ac}$ 32 (0.011)	11 (0.13)	20 (0.06)	3	$DMTrA^{bz}A^{bz}G^{ib}A^{bz}A^{bz}TT^{PF}C^{an}T^{PF}T_{ac}$ 35 (50)	$AAGAATT^{P}CT^{P}T$ (15 °C)

Abbreviations: The internucleotidic phospho groups are omitted. DMTr, 5′-*O*-dimethoxytrityl; an, N^4-anisoyl; ac, 3′-*O*-acetyl; bz, N^6-benzoyl; p, *p*-chlorophenylphosphoryl; (CE), 2-cyanoethyl; ib, N^2-isobutyryl; P, 5-putrescinyl; PF, *N,N′*-ditrifluoroacetylputrescinyl.

TABLE II. Oligodeoxyribonucleotides Prepared by the Solid-Phase Phosphotriester Method, and Their Melting Temperatures (T_m)[a]

Oligonucleotide	T_m	Oligonucleotide	T_m	
AGA-TA-GC-TA-TC-T	46 °C	TTTT-TTTT-TTT-T	29 °C[b]	T_{12}
68 84 60 86 77[c]		61 55 94		
AGA-T^{P}A-GC-TA-TC-T	37 °C	TT-T^{P}TT-TTT-T^{P}TT-T	23.5 °C	$T^{P}_{3,9}$
69 66 77 79 69		87 84 82 87		
AGA-TA-GC-T^{P}A-TC-T	40 °C	TT-T^{P}TT-TT^{P}-TTT-T^{P}-T	18.5 °C	$T^{P}_{3,7,11}$
65 78 81 55 70		85 83 71 75 74		
AGA-TA-GC-TA-T^{P}C-T	37 °C	TT^{P}-TTT^{P}-TTT^{P}-TTT^{P}-T	13.5 °C	$T^{P}_{2,5,8,11}$
70 85 81 78 52		79 74 64 78		
		TT-T^{N}TT-TTT-T^{N}TT-T	20 °C	$T^{N}_{3,9}$
		78 69 82 100		
		TT-T^{O}TT-TTT-T^{O}TT-T	25 °C	$T^{O}_{3,9}$
		80 86 76 92		

a) T_m's were measured in 0.1 M NaCl–0.01 M sodium cacodylate at pH 7.2. b) T_m's of the duplex with poly(dA). c) Yields (%) of the condensation steps as measured in terms of the amount of released dimethoxytrityl groups.

However, as has been reported,[22] nuclease S1 treatment followed by VPDase (with APase) treatment resulted in complete hydrolysis of the modified oligonucleotides to the nucleoside components. Thus, treatment of AGATAGCT^{P}ATCT, for example, with the combination of the nucleases gave dC, dT^{P}, dG, dT and dA in the expected ratio. Other oligonucleotides gave similar results. Nuclease P_1 also cleaved the modified nucleotides but the rates were very low (data not shown). Taking account of the synthetic routes, these results indicate that the oligomers synthesized have the expected sequences. The usual two-dimensional fingerprinting for sequence analysis of natural oligonucleotides[23] was not attempted because of the expected incomplete (non-random) hydrolysis by nuclease P_1 and VPDase.

Melting Temperature Profiles of Oligodeoxynucleotides Containing T^{P}

Dodecathymidylates containing T^{P}, T^{N}, or T^{O} were annealed with poly(dA) and their melting temperatures (T_m) were measured (Table II). The natural poly(dA): T_{12} showed the highest T_m (29 °C) among them. The heteroduplex with $T^{O}_{3,9}$ showed a slight decrease of the T_m (25 °C) whereas that of $T^{N}_{3,9}$ showed a still lower T_m (20 °C). This may be because that the NH proton of the 5-CH_2NHCH_3 function of T^{N} can form a hydrogen bond to the 4-C=O group, thus reducing the hydrogen-bond-forming ability of that carbonyl with the adenine moiety, resulting in decreased duplex stability. The duplex with $T^{P}_{3,9}$ also showed a lower T_m (23.5 °C) as compared to the unmodified duplex but higher than that of poly(dA): $T^{N}_{3,9}$. Thus, the primary amino group at the putrescinyl moiety should have some stabilizing effect.

In general, polyamines and diamines bind to DNA and stabilize it[24] and this stability has been attributed to their binding to the minor groove of the DNA helix by ion-pair formation with the phosphoryl anions of the DNA chain.[25] The unusually high T_m of ΦW-14 DNA has also been explained in terms of a similar effect of the putrescinyl moiety.[9] Thus, the T_m of the poly(dA) : oligo(dT) duplex should be elevated by increasing the number of T^{P} substitutions in place of T. However, the present results show that this is not the case, and as the number of substitutions increased from 2 to 4 the T_m became even lower (poly(dA) : $T^{P}_{3,9}$, 23.5 °C; poly(dA) : $T^{P}_{3,7,11}$, 18.5 °C; poly(dA) : $T^{P}_{2,5,8,11}$, 13.5 °C). Therefore the reported stabilizing effect of the putrescinyl moiety may not be due to ionic interaction with the phosphate anion; instead, interactions with other moieties of DNA such as with base moieties should be taken into account.

Therefore, the T_m's of some self-complementary oligomers containing T^{P} were compared

with those of the unmodified oligomers (Tables I and II). These oligomers are in the usual B-type duplexes as revealed by their circular dichroism (CD) spectra (data not shown). In the case of AGATAGCTATCT, the substitution of T^P for T resulted in lowering of T_m's compared to that of the natural dodecamer (T_m 46 °C). However, there seems a certain sequence dependency, and the dodecamer having a C-residue on the 5'-side of T^P showed the highest T_m (40 °C) among the substituted decamers. In the case of the decamer AAGAATTCTT (Table I), the substituted decamer having T^P on the 3'-side of C showed a higher T_m (27 °C) than the natural decamer (22 °C) while the sequence isomer having T^P on the 5'-side of C showed a lower T_m (16 °C). This again shows that there must be some sequence specificity involved in the thermal stability of DNA resulting from the introduction of T^P residues. In oligomers where two T^P residues are located closer together, the T_m's tend to decrease, as shown by AAGAATT^{P}CT^{P}T (<5 °C).

We cannot yet draw any definite conclusion about the role of the putrescinyl residues of T^P in the thermal stability of DNA of ΦW-14. However, it may be concluded that the presence of the putrescinyl residue does not necessarily stabilize the DNA by electrostatic interaction with the phosphoryl moieties, as do polyamines or diamines. The putrescinyl moiety in T^P is located in the major groove of the DNA helix, whereas diamines are bound in the minor groove of DNAs.[25] Although the possibility of ionic interaction of the putrescinyl moiety with the phosphoryl group is not fully excluded, interactions with the base moieties nearby in the duplexes may be involved in the thermal stability of ΦW-14 DNA.

Experimental

General Methods——The ultraviolet (UV) spectra and T_m's were measured with a Shimadzu UV-240 spectrophotometer equipped with a temperature controller and thermometer. CD spectra were measured on a JEOL J-40 or J-500 spectropolarimeter. NMR spectra were recorded on a JEOL FX-100 or 200 FT spectrometer with tetramethylsilane as an internal standard. All exchangeable protons were confirmed by addition of D_2O. Mass spectra (MS) were measured on a JEOL D-300 spectrometer. Melting points were determined on a Yamato MP-21 micromelting point apparatus and are uncorrected. Thin layer chromatography (TLC) and reversed phase TLC were performed on Merck Kieselgel $60F_{254}$ plates and Merck Kieselgel $60F_{254}$ silanisiert, respectively. Silica gel used for column chromatography was Merck Kieselgel 60 (70—230 mesh). The columns used for HPLC were Merck Lichrosorb RP-18 or Whatman Chromedia LPR-2 on a Hitachi 638-30 liquid chromatograph. VPDase and nuclease S1 were purchased from Boehringer Mannheim Co., *E. coli* APase from Worthington Biochemical Inc., and nuclease P_1 from Yamasa Shoyu Co. Poly(dA) was purchased from Sigma Chemical Co. The deoxyribonucleosides for oligonucleotide synthesis were purchased from Yamasa Shoyu Co.

Monophthaloylputrescine (3) Hydrochloride——4-Phthalimidobutyronitrile[26] (10.0 g, 46.7 mmol) was hydrogenated over PtO_2 (200 mg) in EtOH (500 ml) containing concentrated HCl (10 ml) for 1 week. The catalyst was filtered off, the filtrate was concentrated, and the residue was crystallized from EtOH to give **3** as the hydrochloride (8.8 g, 74%), mp 189—191 °C. ^{1}H-NMR (DMSO-d_6) δ: 8.0 (3H, brs, NH_3^+), 7.89 (4H, s, Pht), 3.57 (2H, t, $PhtNCH_2$), 2.67 (2H, t, N-CH_2), 1.58 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{12}H_{15}ClN_2O_2$: C, 56.58; H, 5.94; Cl, 13.92; N, 11.00. Found: C, 56.38; H, 5.91; Cl, 14.42; N, 10.95.

3',5'-Di-*O*-acetyl-2'-deoxy-5-(phthalimidobutylaminomethyl)uridine (5)——a) A mixture of 3',5'-di-*O*-acetyl-2'-deoxy-5-formyluridine[13a,27] (**2**, 1.64 g, 4.8 mmol), monophthaloylputrescine hydrochloride (**3**, 1.85 g, 7.2 mmol) and triethylamine (2.4 g, 24 mmol) in dioxane (10 ml) was stirred for 2 h. $NaBH_4$ (370 mg, 10 mmol) was added to the solution in an ice-bath. After 30 min, the mixture was concentrated to a half of the initial volume, and 80% AcOH was added to neutralize the solution. The solvent was removed *in vacuo* and the residue was extracted with $CHCl_3$. The organic layer was concentrated and the concentrate was chromatographed on silica gel (1.5 × 40 cm). The eluate with $CHCl_3$–MeOH (10 : 1) was concentrated to leave **5** (1.8 g, 62%) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 7.81, 7.71 (2H each, m, Pht), 6.31 (1H, dd, H-1'), 5.22 (1H, br d, H-3'), 5.0—4.2 (2H, br, NH), 4.34 (2H, dd, H-5'), 4.26 (1H, m, H-4'), 3.70 (2H, t, $PhtNCH_2$), 3.52 (2H, q, 5-CH_2, J_{gem} = 14.2 Hz), 2.67 (2H, t, NCH_2), 2.46, 2.33 (1H each, dd, H-2'), 2.12, 2.11 (3H each, s, Ac). 1.9—1.5 (4H, m, CH_2CH_2).

b) 3',5'-Di-*o*-acetylthymidine (1.0 g, 3.07 mmol) was brominated with Br_2 (0.18 ml, 3.3 mmol) in CCl_4 (200 ml) under irradiation by the reported procedure.[27] The product was dissolved in acetonitrile (40 ml) and treated with monophthaloylputrescine hydrochloride (5.0 g, 19.6 mmol) and K_2CO_3 (4.14 g, 30 mmol) in acetonitrile (70 ml) under cooling in an ice bath for 1 h. The precipitate was filtered off, the filtrate was concentrated, and the residue was chromatographed on silica gel (1.5 × 40 cm) to give **5** (860 mg, 52%). The physical constants were similar to those of

the product obtained in a).

Putrescinylthymidine Mono-oxalate (1)——Compound **5** (860 mg, 1.6 mmol) was dissolved in 40% aqueous $MeNH_2$ (15 ml) and the mixture was kept at room temperature for 3 d. The solvent was removed *in vacuo* and the residue was adsorbed on Amberlite IRC 50 resin (H^+ form, 100 ml). The resin was washed with H_2O (120 ml) and the product was eluted with 0.3 M NH_4OH. The eluate was concentrated to leave an oily residue (500 mg). This was taken up in a small volume of MeOH, and the solution was neutralized with oxalic acid. The precipitate was collected, then crystallized from MeOH, mp 188—190.5 °C (dec.). UV $\lambda_{max}^{H_2O}$ nm (ε): 264 (8800), λ_{max}^{pH1}: 264 (9600), λ_{max}^{pH12}: 264 (5300). ^{1}H-NMR (D_2O) δ: 8.15 (1H, s, H-6), 6.27 (1H, t, H-1', J=6.4 Hz), 4.44 (1H, m, H-3'), 4.07 (1H, m, H-4'), 4.02 (2H, s, 5-CH_2), 3.85 (1H, m, H-5'), 3.08 (4H, m, NCH_2), 2.41 (2H, m, H-2'), 1.78 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{16}H_{26}N_4O_9 \cdot 2/3H_2O$: C, 44.52; H, 6.38; N, 12.98. Found: C, 44.77; H, 6.22; N, 12.95.

2'-Deoxy-5-(*N*-methyl-*N*-trifluoroacetylaminomethyl)uridine (9)——A solution of 3',5'-di-*O*-acetylthymidine (2.0 g, 6.1 mmol) in CCl_4 (400 ml) was treated with Br_2 (0.4 ml, 7 mmol) under irradiation.[27] After 1 h, the solvent was removed *in vacuo* and the residue (5-bromomethyl derivative) was dissolved in CH_3CN (30 ml). A saturated solution of $MeNH_2$ in acetonitrile (30 ml) was added dropwise to the solution in an ice-bath, and the whole was kept for 15 min. The solvent was evaporated off and the residue was taken up in 1.5 M NH_4OH (100 ml). After 1 h at room temperature, the solvent was removed and the residue was absorbed on Dowex 50W × 8 resin (H^+, form, 3 × 30 cm). The column was washed with H_2O, then eluted with 0.5 M NH_4OH. The eluate was concentrated, the residue (1.2 g) was dried by codistillation with pyridine, and the residue was dissolved in MeOH. Ethyl trifluoroacetate (4.3 g, 30 mmol) and triethylamine (3.0 g, 30 mmol) were added and the solution was kept overnight at room temperature. The solvent was evaporated off and the residue was applied to a column of silica gel (1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (15 : 1) was concentrated to leave **9** as a foam (1.3 g, 58%). An aliquot was crystallized from MeOH, mp 159—163 °C. High-resolution MS *m/z*: 367.0992. Calcd ($C_{13}H_{16}F_3N_3O_6$): 367.0999. MS *m/z*: 367 (M^+), 251 (B+H), 117 (M^+ − B). ^{1}H-NMR (D_2O) δ: 7.94, 7.90 (1H, s each, H-6), 6.30 (1H, t, H-1'), 4.50 (1H, m, H-3'), 4.37 (2H, s, 5-CH_2), 4.07 (1H, m, H-4'), 3.82 (2H, m, H-5'), 3.23, 3.21 (3H, s each, NCH_3), 2.39 (2H, m, H-2').

2'-Deoxy-5-(methylaminomethyl)uridine Acetate (6)——A solution of **9** (110 mg, 0.3 mmol) in concentrated NH_4OH (3 ml) was kept at room temperature for 30 min. The solvent was removed *in vacuo* and the residue was dissolved in 10% AcOH (1 ml). The solvent was removed and the residue was crystallized from aqueous MeOH to give **6** (60 mg, 72.3%), mp 113—115 °C. UV $\lambda_{max}^{H_2O}$ nm (ε): 265 (10800); λ_{max}^{pH1}: 265 (9600); λ_{max}^{pH12}: 264 (6400). ^{1}H-NMR (D_2O) δ: 8.14 (1H, s, H-6), 6.28 (1H, t, H-1'), 4.48 (1H, m, H-3'), 4.08 (1H, m, H-4'), 4.00 (2H, s, 5-CH_2), 3.83 (2H, dd, H-5'), 2.73 (3H, s, NCH_3), 2.41 (2H, m, H-2'), 1.92 (3H, s, acetate). *Anal.* Calcd for $C_{13}H_{21}N_3O_7 \cdot 5/4H_2O$: C, 44.12; H, 6.69; N, 11.87. Found: C, 44.35; H, 6.46; N, 11.58.

2'-Deoxy-5'-dimethoxytrityl-5-(methoxymethyl)uridine (12)——3',5'-Di-*O*-acetylthymidine (3.0 g, 9.2 mmol) was monobrominated by the procedure[27] described above and the product was dissolved in 5 M NaOMe in MeOH (20 ml). After 30 min, the solution was neutralized by addition of AcOH. The solvent was removed *in vacuo*, the residue was taken up in MeOH (20 ml), the insoluble material was filtered off, and the filtrate was concentrated to leave a syrup of crude 2'-deoxy-5-(methoxymethyl)uridine (**7**, 2.1 g). Compound **7** (1.0 g, 3.67 mmol) was dissolved in pyridine (2 ml). Dimethoxytrityl chloride (1.24 g, 3.67 mmol) was added to the pyridine solution, and after 1 h at room temperature MeOH (1 ml) was added. The solvent was removed, the residue was taken up in $CHCl_3$, and the organic layer was washed with aqueous $NaHCO_3$ solution and dried over Na_2SO_4. The solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with benzene–AcOEt (1 : 1) was concentrated to leave **12** (39% from the thymidine) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 8.54 (1H, br s, NH), 7.77 (1H, s, H-6), 7.47—7.23, 6.88—6.79 (9H and 4H, m and d, phenyl protons), 6.36 (1H, t, H-1'), 4.51 (1H, m, H-3'), 4.04 (1H, m, H-4'), 3.9—3.75, 3.80 (2H and 6H, m and s, 5-CH_2 and CH_3O-Ph), 3.40 (2H, t, H-5'), 3.06 (3H, s, CH_3O), 2.4—2.1 (2H, m, H-2').

2'-Deoxy-5-(methoxymethyl)uridine (7)——Pure **7** was obtained by deprotection of **12**. Compound **12** (200 mg, 0.35 mmol) was dissolved in 80% AcOH (3 ml). After 30 min, the solvent was removed and the residue was dried by co-distillation with toluene. The residue was partitioned between H_2O and $CHCl_3$. The aqueous layer was concentrated and the residue was crystallized from AcOEt to give **7** (80 mg, 85%), mp 116—117 °C (lit. 120—125 °C[28]). UV $\lambda_{max}^{H_2O}$ nm (ε): 264 (11500); λ_{max}^{pH1}: 264 (10500, lit., 10700); λ_{max}^{pH12}: 264 (9500). MS *m/z*: 272 (M^+), 156 (B+H), 117 (M^+ − B). ^{1}H-NMR (D_2O) δ: 7.98 (1H, s, H-6), 6.29 (1H, t, H-1'), 4.46 (1H, m, H-3'), 4.25 (2H, s, 5-CH_2), 4.04 (1H, m, H-4'), 3.80 (2H, dd, H-5'), 3.37 (3H, s, CH_3O), 2.41 (2H, m, H-2'). *Anal.* requires for $C_{11}H_{16}N_2O_6$: C, 48.52; H, 5.92; N, 10.29. Found: C, 48.28; H, 5.85; N, 10.11.

***N,N'*-Bis(trifluoroacetyl)putrescinylthymidine (8)**——Compound **1** (1.1 g, 2.56 mmol), ethyl trifluoroacetate (3.55 g, 25 mmol) and triethylamine (1.25 g, 12.5 mmol) were dissolved in MeOH (30 ml). After 2 d at room temperature, the solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated and the residue was crystallized from MeOH to give **8** (800 mg, 62.1%), mp 168—170 °C. MS *m/z*: 520 (M^+), 423 (M^+ −97), 404 (B+H), 117 (M^+ − B). ^{1}H-NMR (DMSO-d_6) δ: 11.50, 11.46 (1H, br s each, N^3H), 9.44 (1H, br, NH), 7.84, 7.83 (1H, s each, H-6), 6.13 (1H, m, H-1'), 5.22, 4.99 (1H each, m, HO-5', 3'), 4.24 (1H, br s, H-3'), 4.18 (2H, s, 5-CH_2), 3.78 (1H, m, H-4'), 3.4—3.2, 3.16 (4H and 2H, m each, H-5', N–CH_2 and NCH_2), 2.1 (2H, m, H-2'), 1.8—1.4 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{18}H_{22}F_6N_4O_7$: C, 41.54; H, 4.26; N, 10.77. Found: C, 41.43; H, 4.28; N, 10.81.

5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (10)——Compound **8** (670 mg, 1.3 mmol) was dimethoxytritylated by the usual procedure to give **10** (550 mg, 67%) after chromatographic separation. ^{1}H-NMR ($CDCl_3$) δ: 9.8—9.6 (2H, br, NH), 7.93 (1H, s, H-6), 7.45—7.15, 6.83 (9H and 4H, m and d, Ph), 6.24 (1H, t, H-1′), 4.5 (1H, m, H-3′), 3.95 (1H, m, H-4′), 3.87 (2H, s, 5-CH_2), 3.80, 3.78 (3H each, s, CH_3O), 3.5—3.2 (6H, m, H-5′ and NCH_2), 2.40, 2.27 (1H each, m, H-2′), 1.70 (4H, br s, CH_2CH_2). A small amount (6.8%) of by-product (3′,5′-bis(dimethoxytrityl)derivative) was also obtained as a foam.

3′-*O*-Acetyl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine (13)——Compound **10** (250 mg, 0.3 mmol) in pyridine (3 ml) was treated with Ac_2O (120 mg, 1.2 mmol) at room temperature overnight. The solvent was removed and the residue was partitioned between $CHCl_3$ and $NaHCO_3$–H_2O. The organic layer was dried, the solvent was removed *in vacuo*, and the residue was dessolved in 5 ml of 2% benzensulfonic acid (in $CHCl_3$–MeOH, 7 : 3). After 10 min at room temperature, the solution was diluted with $CHCl_3$, and washed with $NaHCO_3$–H_2O. The dried organic layer was chromatographed (silica gel, 0.9 × 50 cm), the eluate with $CHCl_3$–MeOH (30 : 1) was concentrated, and the residue was crystallized from EtOH to give **13** (100 mg, 60%), mp 141—142 °C. ^{1}H-NMR ($CDCl_3$) δ: 10.16 (1H, s, NH), 8.42 (1H, s, H-6), 7.5 (1H, br s, NH), 6.23 (1H, t, H-1′), 5.39 (1H, m, H-3′), 4.36—4.16 (3H, m, H-4′, CH_2-5), 3.92 (2H, m, H-5′), 3.56—3.37 (4H, m, CH_2–N), 2.46 (2H, m, H-2′), 1.79—1.66 (4H, m, CH_2CH_2). *Anal.* Calcd for $C_{20}H_{24}F_6N_4O_8$: C, 42.71; H, 4.30; N, 9.96. Found: C, 42.29; H, 4.30; N, 10.07.

2′-Deoxy-5′-*O*-dimethoxytrityl-5-(*N*-methyl-*N*-trifluoroacetylaminomethyl)uridine (11)——Compound **9** (330 mg, 0.9 mmol) was dried by co-distillation with pyridine and the residue was dissolved in pyridine (1 ml). Dimethoxytrityl chloride (340 mg, 1 mmol) was added and the solution was kept at room temperature for 1 h. A few drops of MeOH were added, then the solvent was removed *in vacuo*, and the residue was partitioned between $CHCl_3$ and H_2O. The organic layer was concentrated and the residue was applied to a silica gel column (1.2 × 40 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated to leave **11** (456 mg, 76%) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 8.76 (1H, br s, NH), 7.82 (1H, s, H-6), 7.57—7.13 and 6.90—6.78 (9H and 4H, m each, Ph), 6.26 (1H, t, H-1′), 4.45 (1H, m, H-3′), 4.05—3.90 (3H, m, H-4′, CH_2-5), 3.78 (6H, s, OCH_3), 3.42 (2H, d, H-5′), 3.24, 3.22 (3H, s each, CH_3–N), 2.23 (2H, m, H-2′), 2.15 (1H, br s, HO-3′).

5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl 2-cyanoethyl)phosphate (14)——Compound **10** (520 mg, 0.63 mmol) was dried by co-distillation with pyridine and the residue was dissolved in pyridine (2 ml). *p*-Chlorophenyl phosphoroditriazolide (0.1 M in tetrahydrofuran, 8 ml)[15] was added to the solution. After 30 min, *N*-methylimidazole (308 mg, 3.75 mmol) and 2-cyanoethanol (438 mg, 6.25 mmol) were added and the solution was concentrated to a half of its original volume, then kept for 1.5 h at room temperature. The reaction was quenched by addition of a small volume of H_2O under cooling in an ice bath, and the solution was extracted with $CHCl_3$. The organic layer was washed with $NaHCO_3$–H_2O, then dried over Na_2SO_4, and the solvent was removed. The residue was applied to a silica gel column (10 × 50 cm). The eluate with $CHCl_3$–MeOH (20 : 1) was concentrated to leave **14** (590 mg, 88%). ^{1}H-NMR ($CDCl_3$) δ: 9.0 (1H, br s, N^3H), 8.64 (1H, br s, NH), 7.96 (1H, s, H-6), 7.69—7.03 and 6.81 (9H and 4H, m and d, Ph), 6.23 (1H, t, H-1′), 5.1 (1H, m, H-3′), 4.32—4.18 (4H, m, CH_2-5, CH_2CH_2CN), 3.85 (1H, br s, H-4′), 3.77 (6H, s, CH_3O), 3.7—3.2 (6H, m, H-5′ and CH_2N), 2.70—2.58 (2H, m, CH_2CN), 2.42 (2H, m, H-2′), 1.61 (4H, br s, CH_2CH_2).

***N*,*N*′-Bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl 2-cyanoethyl)phosphate (15)**——Compound **14** (prepared from 0.5 mmol of **10**) was dissolved in a 2% solution of benzenesulfonic acid ($CHCl_3$–MeOH, 7 : 3, 5 ml). After 20 min, the solution was diluted with $CHCl_3$ and washed with 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4. The solvent was removed and the residue was chromatographed (silica gel, 1.5 × 50 cm). The eluate with $CHCl_3$–MeOH (20 : 1) was concentrated to leave **15** (290 mg, 59.4% from **10**). This was used immediately for the next step.

Triethylammonium 5′-*O*-Dimethoxytrityl-*N*,*N*′-bis(trifluoroacetyl)putrescinylthymidine 3′-(4-Chlorophenyl)-phosphate (16)——a) Compound **10** (400 mg, 0.48 mmol) in dioxane (1 ml) was treated with *p*-chlorophenyl-phosphoroditriazolide (0.1 M in dioxane, 3.5 ml) at room temperature for 1.5 h. Aqueous pyridine (90%, 1 ml) was added and the mixture was concentrated to a volume of *ca.* 2 ml. This was extracted with $CHCl_3$ and the organic layer was washed with 0.1 M triethylammonium bicarbonate. Pyridine was added to the solution, the solvent was removed *in vacuo* and the residue was dried by co-distillation with pyridine several times to leave **16** (480 mg, 91%). This was used for the next step.

b) Compound **14** (590 mg, 0.55 mmol) in pyridine (3 ml) was treated with triethylamine (1.0 g, 10 mmol) at room temperature for 4 h. The solvent was removed *in vacuo* and the residue was dried by co-distillation with pyridine to leave **16**, which was stored at −20 °C until use.

Synthesis of TT^P and T^{P}T——A solution of the triethylammonium salt of 5′-*O*-dimethoxytritylthymidine 3′-(*p*-chlorophenyl)phosphate (96 mg, 0.12 mmol) and **13** (56 mg, 0.10 mmol) in pyridine (1 ml) was treated with TPSCl (156 mg, 0.5 mmol) and *N*-methylimidazole (82 mg, 1 mmol) at room temperature for 1 h. The reaction was quenched by addition of H_2O (0.1 ml) in an ice bath, and $CHCl_3$ was added. The organic layer was washed with 5% $NaHCO_3$ and H_2O, then dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue was applied to a column of silica gel (0.9 × 50 cm). The eluate with $CHCl_3$–MeOH (30 : 1) was concentrated to leave the protected dimer (130 mg, 93%) as a foam. The foam was taken up in conc. NH_4OH (15 ml) and pyridine (5 ml), and the mixture was kept at room temperature for 12 h, then at 55 °C for 4 h. The solvent was removed *in vacuo* and the residue was dissolved in 80%

AcOH (5 ml). After 1 h at toom temperature, the solvent was removed *in vacuo*, and the residue was co-distilled with toluene to remove a trace of AcOH. The residue was dissolved in 0.1 M triethylammonium bicarbonate and the solution was washed with EtOAc several times, then concentrated. The residue was applied to a column of Amberlite IRC 50 (H^+) resin (1 × 40 cm) and the column was washed with H_2O, then eluted with 0.3 M NH_4OH. The eluate (A_{260} > 1) was concentrated and the residue was chromatographed on DEAE-cellulose. The eluate with 0.1 M triethylammonium bicarbonate was concentrated to leave TT^P (TOD_{260} = 1100). ^1H-NMR (D_2O, the number 1 in parentheses indicates the 5′-nucleoside protons and 2, the 3′-nucleoside protons) δ: 8.00 (1H, s, H-6(2)), 7.65 (1H, s, H-6(1)). TT^P was not hydrolyzed by VPDase and APase (37 °C, 3 d) as checked by HPLC. T^PT was prepared by a similar method. The diester (125 mg, 0.12 mmol) from **14** and 3′-*O*-acetylthymidine (39 mg, 0.1 mmol) were condensed to give the protected dimer (140 mg, quant.), which was deprotected to leave T^PT (TOD_{260} = 1030). Digestion with VPDase and APase (37 °C, 3 d) gave dT and dT^P (T/T^P = 1.05). ^1H-NMR (D_2O): δ: 7.85 (1H, s, H-6(2)), 7.69 (1H, s, H-6(1)), 6,24, 6.23 (1H each, t, H-1′), 4.67 (1H, m, H-3′(2)), 4.59—4.00 (4H, m, H-4′(1, 2), H-5′(1)), 3.83—3.69 (5H, m, CH_2-5, H-3′(1), H-5′(2)), 3.01, 2.83 (2H each, m, CH_2N), 2.6—2.4, 2.35—2.1 (1H and 3H, m, H-2′(1, 2)), 1.90 (3H, s, CH_3(2)), 1.70 (4H, m, CH_2CH_2).

Synthesis of Oligomers by the Liquid-Phase Phosphotriester Method——The following oligomers were prepared by liquid-phase oligonucleotide-block condensation *via* phosphotriester method[16,17] starting from **15** or **16** for introduction of T^P: TTCTT, TT^PCTT, TT^PCT^PT, AAGAATTCTT, $AAGAATTT^PCTT$, $AAGAATTCT^PT$, and $AAGAATT^PCT^PT$.

Synthesis of Dodecanucleotides by the Solid-Phase Phosphotriester Method——The following dodecanucleotides were prepared by the solid phase phosphotriester method developed by Itakura *et al.*[20] on aminomethylpolystyrene as the polymer support: AGATAGCTATCT, $AGAT^PAGCTATCT$, $AGATAGCT^PATCT$, $AGATAGCTAT^PCT$, TTTTTTTTTTTT, $TT^PTTT^PTTT^PT$, $TTT^PTTTT^PTTTT^PT$, $TTT^PTTTTTT^PTTT$, $TTT^NTTTTTT^NTTT$, and $TTT^OTTTTTT^OTTT$.

Annealing of Poly(dA) : Oligo(dT)——Poly(dA) (0.86 OD_{265}/ml) and oligo(dT) containing T^P, T^N or T^O (0.89 OD_{265}/ml) were annealed in 0.1 M NaCl–0.01 M sodium cacodylate (pH 7.2) by the reported procedure.[29]

Digestion of Oligothymidylates by Nuclease S1——Oligothymidylate or an analog containing T^P, T^N, and T^O (0.5—1.0 OD unit) was incubated in a buffer (50 mM $AcONH_4$, 50 mM NaCl, 5 mM $ZnSO_4$, pH 5.0, 60 μl) with nuclease S1 (5 × 10^5 unit/ml, 10 μl) at 50 °C overnight. The hydrolyzate was analyzed by HPLC. In the case of T_{12}, the molar ratio of 5′-TMP and thymidine was 1.0 : 10.8.

Digestion of Oligothymidylates by VPDase——The dodecathymidylate containing T^P at positions 3 and 9, T^N at positions 3 and 9, or T^O at positions 3 and 9 (*ca.* 2 OD units) was incubated with VPDase (1 mg/ml, 10 μl) and bacterial alkaline phosphatase (360 units/ml, 5 μl) in a buffer (100 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 8.9) at 37 °C for 15 h. The hydrolyzate was analyzed by HPLC.

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Homoisoflavonoids and Related Compounds. III.[1] Phenolic Constituents of *Caesalpinia japonica* SIEB. *et* ZUCC.

MICHIO NAMIKOSHI,* HIROYUKI NAKATA, MARIKO NUNO, TAKASHI OZAWA and TAMOTSU SAITOH

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

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A new homoisoflavonoid, 3'-deoxy-4-*O*-methylsappanol (3,7-dihydroxy-3-(4-hydroxybenzyl)-4-methoxychroman) was isolated from the wood of *Caesalpinia japonica* SIEB. *et* ZUCC. (Leguminosae), together with the known compounds, sappanol, episappanol, 4-*O*-methylsappanol, 4-*O*-methylepisappanol, sappanones A and B, sappanchalcone, 3-deoxysappanchalcone, isoliquiritigenin, butein, brazilin and protosappanins A, B and C. None of these compounds has previously been isolated from this plant.

Keywords——*Caesalpinia japonica*; Leguminosae; wood; isolation; homoisoflavonoid; brazilin; dibenzoxocin derivative

As a part of our studies on homoisoflavonoids and related compounds, we have investigated the chemical constituents of Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L., and reported the isolation and structural elucidation of several homoisoflavonoidal compounds.[1-3] Homoisoflavonoids[4] have been isolated from several genera in Liliaceae and Caesalpinioideae (Leguminosae).[5] The homoisoflavonoidal skeleton consists of sixteen carbons as against fifteen carbons in an isoflavonoidal skeleton. The sixteenth carbon is derived from a methoxy methyl group of a 2'-methoxychalcone, which is an important biogenetic precursor of homoisoflavonoidal compounds, as indicated by the result of an isotope-labeling study by Dewick.[6] He also proposed a biogenetic route to brazilin from 2'-methoxychalcone *via* homoisoflavonoids. Some homoisoflavonoidal precursors for brazilin biosynthesis were proposed by us in previous papers.[1,3]

Brazilin[7] has been isolated as a main component from the heartwood of *C. sappan* and also from the heartwood of *Caesalpinia echinata* LAM. (Brazil wood). These plants have been used as a red dyestuff. The dried heartwood of *C. sappan*, Sappan Lignum, contains several types of phenolic components, that is 2'-methoxychalcones,[2,8] homoisoflavonoids,[1-3] dibenzoxocin derivatives,[9] brazilins,[1,3] and so on.[10] We are interested in the chemotaxonomic relationship between *C. sappan* and other *Caesalpinia* spp.

Caesalpinia japonica (=*C. sepiaria* ROXB. var. *japonica* MAKINO; Japanese name: jaketsuibara) is a deciduous twisted tree growing at riverside locations and forest edges in Japan. The dried seed and root of this plant have been used as an insecticide and a binding medicine, and have been prescribed as a febrifuge of malarial fever in traditional Chinese medicine.[11] Imamura *et al.* studied the chemical components of this plant, and reported the isolation of apigenin, 4',7-dihydroxyflavone, palmitic acid, (+)-pinitol and sitosterol from the stem, and heptacosane, nonacosane, β-carotene, arachic acid, heperin and 1-*O*-(*p*-hydroxybenzoyl)-β-D-glucose from the flower.[12] Their attempt to verify the chemotaxonomic relationship with *C. sappan* was unsuccessful, but this might be ascribed to the plant material they used. We suspected that they had probably collected slender stems. Therefore we

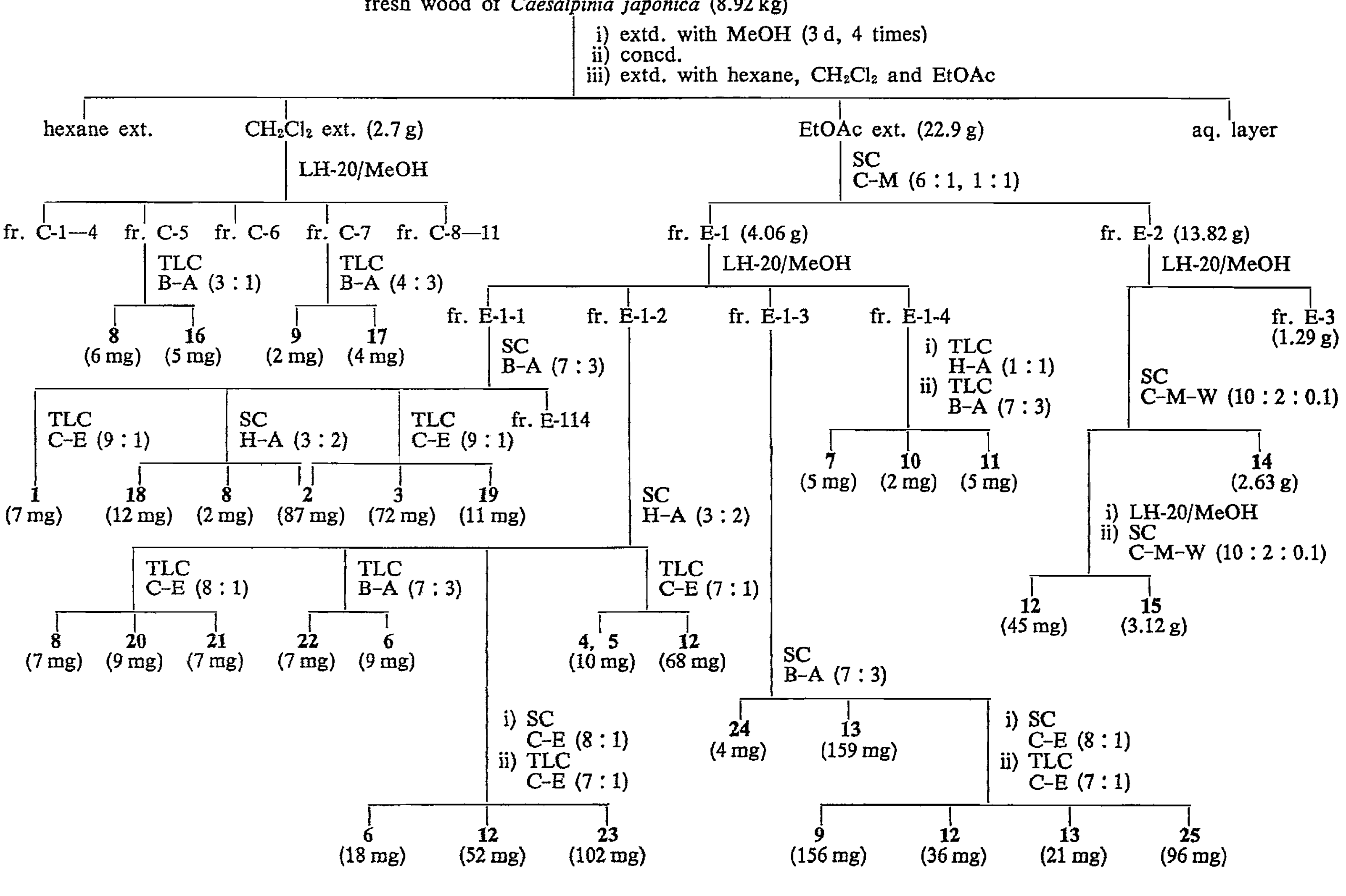

Chart 1

investigated the chemical constituents of the thick wood (3—6 cm in diameter without the bark) of this plant.

In the present paper, we report the isolation and structure assignment of a new homoisoflavonoid, 3′-deoxy-4-*O*-methylsappanol (**1**), and the known compounds 4-*O*-methylsappanol (**2**), 4-*O*-methylepisappanol (**3**), sappanol (**4**), episappanol (**5**), newly named sappanones A (**6**) and B (**7**), 3-deoxysappanchalcone (**8**), sappanchalcone (**9**), isoliquiritigenin (**10**), butein (**11**), brazilin (**12**) and protosappanins A (**13**), B (**14**) and C (**15**).

Extraction and Separation

The fresh wood of *C. japonica* was stripped of bark and cut into small chips. Extraction and separation were carried out as shown in Chart 1.

3′-Deoxy-4-*O*-methylsappanol (1)

3′-Deoxy-4-*O*-methylsappanol (**1**) was obtained as colorless needles, mp 100—101 °C, $[\alpha]_D^{25}$ +40.7° (MeOH). The high-resolution mass spectrum (MS) of **1** revealed the molecular formula, $C_{17}H_{18}O_5$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** showed an aliphatic methoxy methyl singlet at δ 3.33, geminal coupled (J=10.2 Hz) methylene signals at δ 3.89 and 3.63, further long-range-coupled (J=1.3 Hz) with a methoxymethine proton signal at δ 3.68, and characteristic ABX-type aromatic proton signals at δ 6.36 (1H, d, J=2.2 Hz), 6.44 (1H, dd, J=2.2, 8.2 Hz) and 7.05 (1H, d, J=8.2 Hz). These signals closely resembled those of 4-*O*-methylsappanol (**2**) obtained from Sappan Lignum[3,13] and were therefore assignable to the protons at 4-OMe, C-2, C-2, C-4, C-8, C-6 and C-5 positions, respectively (Table I). In addition, a two-proton singlet at δ 2.60 and a pair of two-proton doublets (J=9.0 Hz) at δ 6.70 and 6.97 indicated the presence of a *p*-hydroxybenzyl group.

Consequently, the structure of 3′-deoxy-4-*O*-methylsappanol (**1**) should be represented by 3,7-dihydroxy-3-(4-hydroxybenzyl)-4-methoxychroman.

The absolute configuration at the C-3 and C-4 positions of **1** was elucidated by the comparison of its circular dichroism (CD) spectrum with that of 3-deoxysappanol[1,2] and its 4′,7-dimethyl ether.[1] A negative Cotton effect was observed in the CD curves of **1**, 3-deoxysappanol and its 4′,7-dimethyl ether as shown in Fig. 1. These CD experiments revealed the absolute configuration at the C-3 and C-4 positions of 3′-deoxy-4-*O*-methylsappanol (**1**) to be (3*R*,4*S*) (Chart 2).

TABLE I. ^{1}H-NMR Data for Compounds **1**, **2** and **3**[a]

	1 (Acetone-d_6)		**2** (Acetone-d_6)		**3** (Acetone-d_6)	
H-2	3.63 dd	J=1.3, 10.2	3.62 br d	J=10.2	3.80 dd	J=1.6, 11.0
	3.89 d	J=10.2	3.89 d	J=10.2	4.10 d	J=11.0
H-4	3.68 br s		3.69 br s		3.66 br s	
H-5	7.05 d	J=8.2	7.04 d	J=8.2	6.96 d	J=8.2
H-6	6.44 dd	J=2.2, 8.2	6.44 dd	J=2.2, 8.2	6.34 dd	J=2.4, 8.2
H-8	6.36 d	J=2.2	6.35 d	J=2.2	6.28 d	J=2.4
H-9	2.60 s		2.56 s		2.63 d	J=14.0
					2.89 d	J=14.0
H-2′	6.97 d	J=9.0	6.70 d	J=2.0	6.85 d	J=1.8
H-6′			6.44 dd	J=2.0, 8.0	6.64 dd	J=1.8, 8.0
H-5′	6.70 d	J=9.0	6.68 d	J=8.0	6.76 d	J=8.0
H-3′			—		—	
4-OMe	3.33 s		3.33 s		3.31 s	

a) Chemical shifts are given in δ (ppm) relative to tetramethylsilane. Coupling constants are given in Hz.

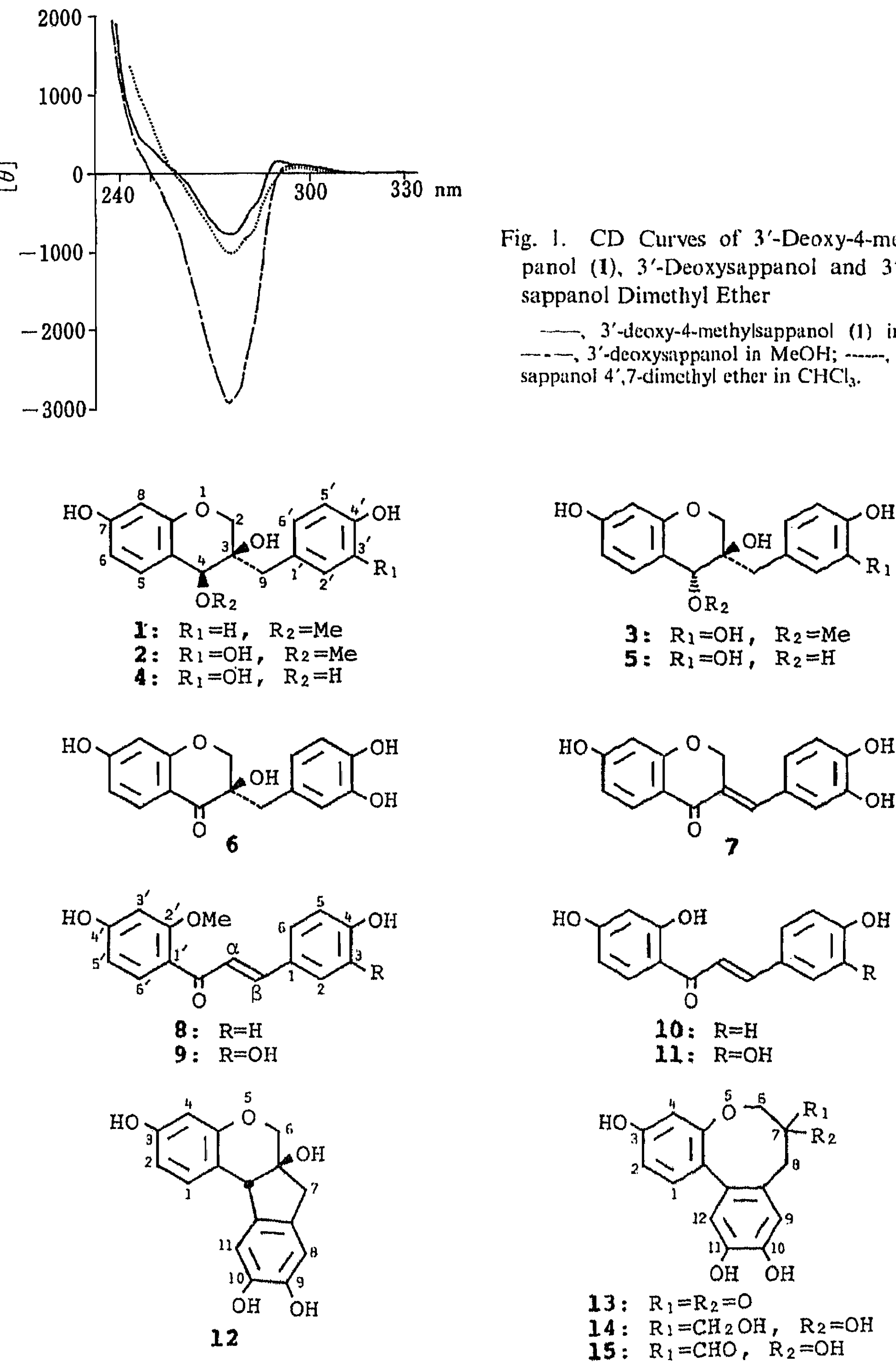

Fig. 1. CD Curves of 3′-Deoxy-4-methylsappanol (1), 3′-Deoxysappanol and 3′-Deoxysappanol Dimethyl Ether

——, 3′-deoxy-4-methylsappanol (1) in MeOH; ---—, 3′-deoxysappanol in MeOH; ------, 3′-deoxysappanol 4′,7-dimethyl ether in $CHCl_3$.

Chart 2

Structural Assignment of Known Compounds

4-*O*-Methylsappanol (2) has a specific rotation of +54.4° (MeOH). The molecular ion peak was not detected in the electron ionization MS (EI-MS), but an $M^+ - MeOH$ ion peak was observed at *m*/*z* 286, which corresponds to the M^+ ion peak for brazilin (**12**). 4-*O*-Methylepisappanol (3), $[\alpha]_D^{25}$ −36.2° (MeOH), showed an $M^+ - MeOH$ ion peak at *m*/*z* 286 in its EI-MS, as in the case of **2**. Compounds **2** and **3** were readily transformed into brazilin (**12**) upon heating in MeOH in the presence of an acid.[3,13] The ^1H-NMR spectra of **2** and **3**

(Table I) showed a slight difference from each other. Compounds **2** and **3** were therefore deduced to be stereoisomers at the C-4 position. The physicochemical properties of **2** and **3** were identical with those of 4-*O*-methylsappanol and 4-*O*-methylepisappanol obtained from Sappan Lignum,[3,14] respectively. Accordingly, compounds **2** and **3** are (3*R*,4*S*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)-4-methoxychroman and (3*R*,4*R*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)-4-methoxychroman,[13] respectively, as shown in Chart 2.

Sappanol (**4**) and episappanol (**5**) were obtained as a mixture of the epimers, as in the case of the separation from Sappan Lignum.[1] The mixture of **4** and **5** gave brazilin (**12**) upon acid treatment.[1] The physicochemical properties of the mixture of **4** and **5** were identical with those of the mixture of sappanol and episappanol obtained from Sappan Lignum[1,3,14] (compound **4** in ref. 3). Sappanol (**4**) and episappanol (**5**) are therefore (3*R*,4*S*)-3-(3,4-dihydroxybenzyl)-3,4,7-trihydroxychroman[1] and (3*R*,4*R*)-3-(3,4-dihydroxybenzyl)-3,4,7-trihydroxychroman,[1] respectively (Chart 2).

Interestingly, the ^{1}H-NMR signals due to the methylene protons at the C-9 position of 3,4-*cis* compounds, such as sappanol (**4**), 3′-deoxysappanol,[1,2] 3′-*O*-methylsappanol,[1] 4-*O*-methylsappanol (**2**) and 3′-deoxy-4-*O*-methylsappanol (**1**), were observed as a singlet (measured at 100 MHz), while the signals due to the C-9 methylene protons of 3,4-*trans* compounds, such as episappanol (**5**), 3′-deoxyepisappanol,[1] 3′-*O*-methylepisappanol[1] and 4-*O*-methylepisappanol (**3**), showed a typical AA′ geminal coupling. The characteristics of these signals are useful for the stereochemical assignment of 3,4-di-*O*-substituted homoisoflavans.[13]

Sappanone B (**6**), $[\alpha]_D^{25}$ $+53.7°$ (MeOH), possesses the molecular formula, $C_{16}H_{14}O_6$ (high-resolution MS). The physical and spectral properties of **6** were identical with those of sappanone B obtained from Sappan Lignum[3,14] (compound **3** in ref. 3). The structure of **6** is therefore (3*R*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)-chroman-4-one,[13] as shown in Chart 2.

Sappanone A (**7**), $C_{16}H_{12}O_5$, was obtained as yellow needles, mp 220—221 °C. The physicochemical properties of **7** were identical with those of an authentic sample[14] (compound **2** in ref. 3). Accordingly, sappanone A (**7**) is 3-(3,4-dihydroxybenzylidene)-7-hydroxychroman-4-one (Chart 2).

3-Deoxysappanchalcone (**8**) was obtained as yellow needles, mp 210—212 °C. The spectral data of **8** were superimposable on those of 4,4′-dihydroxy-2′-methoxychalcone[2,15] (Chart 2) obtained from Sappan Lignum[14] (compound **1** in ref. 2).

Sappanchalcone (**9**) was isolated as yellow needles, mp 198—199 °C, and has the molecular formula, $C_{16}H_{14}O_5$ (high-resolution MS). The ^{1}H-NMR spectrum of **9** exhibited an aromatic methoxy methyl singlet at δ 3.92, characteristic ABX-type aromatic proton signals and ABC-type aromatic proton signals. In addition, it showed the presence of an α,β-unsaturated ketone (signals at δ 7.48 and 7.56). These data and the other physicochemical properties of **9** were identical with those of sappanchalcone[8] isolated from Sappan Lignum.[14] Sappanchalcone (**9**) is therefore 2′-methoxy-3,4,4′-trihydroxychalcone as shown in Chart 2.

Isoliquiritigenin (**10**),[16,17] yellow needles, mp 200—202 °C and butein (**11**),[16,18] yellow needles, mp 211—213 °C were identified by comparison with authentic samples.[14]

Brazilin (**12**), $[\alpha]_D^{25}$ $+93.7°$ (MeOH), has the molecular formula, $C_{16}H_{14}O_5$ (high resolution MS). The physical and spectral properties were identical with those of brazilin[1,7] isolated from Sappan Lignum.[14] The structure of **12** is therefore (6a*S*,11b*R*)-3,6a,9,10-tetrahydroxy-6a,11b-dihydro-7*H*-indeno[2,1-*c*]chromene (Chart 2).

Protosappanin A (**13**) was isolated as colorless needles, mp 254—257 °C. The molecular formula, $C_{15}H_{12}O_5$, was deduced from its high-resolution MS. The ^{1}H-NMR spectrum of **13** showed two aliphatic methylene singlets at δ 3.44 and 4.49, and aromatic proton signals at δ 7.16 (1H, d, J=9.0 Hz) and 6.65—6.90 (4H, m). These physicochemical data and the other

spectral properties indicated that **13** is identical with protosappanin A[9a] isolated from Sappan Lignum. Accordingly, compound **13** is 7,8-dihydro-3,10,11-trihydroxy-6*H*-dibenz[*b*,*d*]oxocin-7-one[14] (Chart 2).

Protosappanin B (**14**), $[\alpha]_D^{25}$ $-12.5°$ (MeOH), showed M^+, $C_{16}H_{16}O_6$, at *m/z* 304 in the MS. The ^{1}H-NMR spectrum of **14** showed broadening of the signals measured at room temperature. The ultraviolet (UV) spectrum of **14** exhibited two absorption maxima at 288 and 254 nm, which were suggestive of a 7,8-dihydro-6*H*-dibenzoxocin skeleton. The physicochemical properties of **14** were identical with those of protosappanin B[9b] obtained from Sappan Lignum. To verify this, compound **14** was converted into its peracetate with Ac_2O in Et_3N/benzene in the presence of 4-dimethylaminopyridine. The physical and spectral properties of **14**-pentaacetate coincided with those of protosappanin B-pentaacetate.[14] Consequently, protosappanin B (**14**) is 7,8-dihydro-3,7,10,11-tetrahydroxy-6*H*-dibenz-[*b*,*d*]oxocin-7-methanol, as shown in Chart 2.

Protosappanin C (**15**), $[\alpha]_D^{25}$ $-37.0°$ (MeOH), has the molecular formula, $C_{16}H_{14}O_6$ (high resolution MS). The UV spectrum of **15** showed absorption maxima at 288 and 254 nm, which revealed the presence of a 7,8-dihydro-6*H*-dibenzoxocin skeleton. The ^{1}H-NMR spectrum of **15** exhibited an aldehyde proton singlet at δ 9.68, and broadening of the other signals. Compound **15**, on $NaBH_4$ reduction in MeOH afforded protosappanin B (**14**), whose physicochemical properties coincided with those of **14**. Accordingly, protosappanin C (**15**) is 7,8-dihydro-3,7,10,11-tetrahydroxy-6*H*-dibenz[*b*,*d*]oxocin-7-carbaldehyde[9c,14] (Chart 2).

These fourteen compounds have not previously been isolated from this plant, and this is only the second report on the isolation of **2**—**7**, **9**, **13**, **14** and **15** as natural products.

The probable precursors for brazilin biosynthesis, sappanchalcone (**9**), sappanones A (**7**) and B (**6**), sappanol (**4**), episappanol (**5**), 4-*O*-methylsappanol (**2**) and 4-*O*-methylepisappanol (**3**), were also isolated from *C. japonica* along with brazilin itself. However, 3-deoxysappanone B, which has been isolated from Sappan Lignum[3] together with the above homoisoflavonoidal components, was not detected in *C. japonica*. It seems unlikely that 3-deoxysappanone B is a significant precursor for brazilin biosynthesis.

Two compounds (**18** and **19** in Chart 1), whose structures have not yet been determined exactly, are unstable and gradually decompose during separation procedures, but are candidates as biogenetic precursors of dibenzoxocin derivatives. The biosynthetic route to dibenzoxocin compounds is under investigation, and the structures of these two compounds will be reported elsewhere.

From the results in our series of studies on the chemical constituents of *C. sappan* and *C. japonica*, it is clear that the wood of *C. japonica* contains several phenolic components in common with *C. sappan*. *Caesalpinia sappan* contains a large quantity of brazilin (**12**) in comparison with the dibenzoxocin compounds,[3] while *C. japonica* afforded a very small amount of brazilin (**12**) and a large amount of dibenzoxocin derivatives. The slender stems of *C. japonica* did not contain homoisoflavonoids, dibenzoxocin derivatives or brazilin, but chalcones and flavonoids were isolated.[19] On the other hand, homoisoflavonoids, dibenzoxocin derivatives and brazilin are produced in the thick wood of *C. japonica*. Further investigations are in progress.

Experimental

General——See part II of this series.[1]

Extraction and Isolation——*Caesalpinia japonica* SIEB. *et* ZUCC. was collected at Sagamiko, Kanagawa Prefecture, in June, 1986 (just after flowering). After removal of the bark, the fresh wood (3—6 cm in diameter) was chipped and quickly steeped into MeOH. The extraction and separation procedures are summarized in Chart 1.

3'-Deoxy-4-*O*-methylsappanol (1)——Colorless neeldes, mp 100—101 °C. $[\alpha]_D^{25}$ $+40.7°$ ($c=0.37$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 284 (3.51), 277 (3.57), 224 (4.08). EI-MS *m/z*: 302.1161 (M^+, Calcd for $C_{17}H_{18}O_5$: 302.1155), 272,

153, 123, 107. CD (c=0.00025 mol/l, MeOH) $[\theta]^{25}$ (nm): +165 (290), −403 (283), −785 (275), +1610 (240). ^{1}H-NMR data are summarized in Table I.

4-*O*-Methylsappanol (2)[3,13]——$[\alpha]_D^{25}$ +54.4° (c=0.62, MeOH). UV λ_{max}^{MeOH} nm (log ε): 284 (3.71), 279 (3.72), 220 (4.22). EI-MS m/z: 286 (M^+ − MeOH), 268, 267, 229. ^{1}H-NMR data are summarized in Table I.

4-*O*-Methylepisappanol (3)[3,13]——$[\alpha]_D^{25}$ −36.2° (c=0.58, MeOH). UV λ_{max}^{MeOH} nm (log ε): 284 (3.89), 280 (3.97), 220 (4.33). EI-MS m/z: 286 (M^+ − MeOH), 268, 267, 229, 153, 123. ^{1}H-NMR data are summarized in Table I.

Sappanol (4) and Episappanol (5)[1,3]——These compounds were obtained as a mixture, as in the case of the separation from *C. sappan*. The spectral data for each compound (synthetic samples) were reported in the preceding paper.[1]

Sappanone B (6)[3,13]——$[\alpha]_D^{25}$ +53.7° (c=0.41, MeOH). UV λ_{max}^{MeOH} nm (log ε): 310 (4.34), 276 (4.65), 230 (4.62). EI-MS m/z: 302.0783 (M^+, Calcd for $C_{16}H_{14}O_6$: 302.0788), 180, 179, 151, 137, 123. ^{1}H-NMR (acetone-d_6) δ: 2.85 (2H, s, H-9), 4.05 (1H, d, J=11.5 Hz, H-2), 4.23 (1H, d, J=11.5 Hz, H-2), 6.48 (1H, d, J=2.2 Hz, H-8), 6.56 (1H, dd, J=2.0, 8.0 Hz, H-6′), 6.64 (1H, dd, J=2.2, 8.8 Hz, H-6), 6.76 (1H, d, J=8.0 Hz, H-5′), 6.81 (1H, d, J=2.0 Hz, H-2′), 7.73 (1H, d, J=8.8 Hz, H-5).

Sappanone A (7)[3]——Yellow needles, mp 220—221 °C. UV λ_{max}^{MeOH} nm (log ε): 369 (4.35), 310 (4.15), 258 (4.12). EI-MS m/z: 284.0686 (M^+, Calcd for $C_{16}H_{12}O_5$: 284.0682), 272, 268, 255, 237, 175. ^{1}H-NMR (CD_3OD) δ: 5.39 (2H, d, J=1.8 Hz, H-2), 6.34 (1H, d, J=2.2 Hz, H-8), 6.55 (1H, dd, J=2.2, 8.9 Hz, H-6), 6.60—7.00 (3H, m, H-2′, 5′ and 6′), 7.69 (1H, t, J=1.8 Hz, H-9), 7.83 (1H, d, J=8.9 Hz, H-5).

3-Deoxysappanchalcone (8)[2,15]——Yellow needles, mp 210—212 °C. UV λ_{max}^{MeOH} nm (log ε): 349 (3.89), 235 (4.04). EI-MS m/z: 270.0865 (M^+, Calcd for $C_{16}H_{14}O_4$: 270.0890), 255, 242, 164, 151, 147, 121, 119, 107. ^{1}H-NMR (acetone-d_6) δ: 3.92 (3H, s, 2′-OMe), 6.54 (1H, dd, J=2.2, 8.5 Hz, H-5′), 6.61 (1H, d, J=2.2 Hz, H-3′), 6.92 (2H, d, J=8.8 Hz, H-3 and 5), 7.44 (1H, d, J=16.2 Hz, H-α), 7.58 (2H, d, J=8.8 Hz, H-2 and 6), 7.62 (1H, d, J=16.2 Hz, H-β), 7.63 (1H, d, J=8.5 Hz, H-6′).

Sappanchalcone (9)[8]——Yellow needles, mp 198—199 °C (lit.[8] 199.5—200.5 °C). UV λ_{max}^{MeOH} nm (log ε): 362 (4.41), 250 (3.73). EI-MS m/z: 286.0832 (M^+, Calcd for $C_{16}H_{14}O_5$: 286.0841), 271, 258, 164, 163, 151, 137, 135, 123. ^{1}H-NMR (acetone-d_6) δ: 3.92 (3H, s, 2′-OMe), 6.56 (1H, dd, J=2.2, 8.5 Hz, H-5′), 6.61 (1H, d, J=2.2 Hz, H-3′), 6.92 (1H, d, J=8.2 Hz, H-5), 7.12 (1H, dd, J=2.0, 8.2 Hz, H-6), 7.26 (1H, d, J=2.0 Hz, H-2), 7.48 (1H, d, J=16.2 Hz, H-α), 7.56 (1H, d, J=16.2 Hz, H-β), 7.67 (1H, d, J=8.5 Hz, H-6′).

Isoliquiritigenin (10)[16]——Yellow needles, mp 200—202 °C (lit.[17] 202—203 °C). EI-MS m/z: 256 (M^+), 255, 239, 228, 227, 163, 150, 137, 120, 107, 91, 81, 69, 65. ^{1}H-NMR (CD_3OD) δ: 6.30 (1H, d, J=2.4 Hz, H-3′), 6.43 (1H, dd, J=2.4, 9.0 Hz, H-5′), 6.86 (2H, d, J=8.8 Hz, H-3 and 5), 7.60 (1H, d, J=15.5 Hz, H-α), 7.63 (2H, d, J=8.8 Hz, H-2 and 6), 7.84 (1H, d, J=15.5 Hz, H-β), 7.97 (1H, d, J=9.0 Hz, H-6′).

Butein (11)[16]——Yellow needles, mp 211—213 °C (lit.[18] 214—215 °C). UV λ_{max}^{MeOH} nm (log ε): 383, 260. EI-MS m/z: 272 (M^+), 163, 137. ^{1}H-NMR (acetone-d_6) δ: 6.36 (1H, d, J=2.4 Hz, H-3′), 6.47 (1H, dd, J=2.4, 9.0 Hz, H-5′), 6.91 (1H, d, J=8.5 Hz, H-5), 7.23 (1H, dd, J=2.0, 8.5 Hz, H-6), 7.35 (1H, d, J=2.0 Hz, H-2), 7.70 (1H, d, J=15.5 Hz, H-α), 7.77 (1H, d, J=15.5 Hz, H-β), 8.12 (1H, d, J=9.0 Hz, H-6′).

Brazilin (12)[1]——$[\alpha]_D^{25}$ +93.7° (c=1.44, MeOH). UV λ_{max}^{MeOH} nm (log ε): 288 (4.04). EI-MS m/z: 286.0843 (M^+, Calcd for $C_{16}H_{14}O_5$: 286.0841), 268, 267, 229. ^{1}H-NMR (acetone-d_6) δ: 2.80 (1H, d, J=16.0 Hz, H-7), 3.06 (1H, d, J=16.0 Hz, H-7), 3.72 (1H, d, J=11.5 Hz, H-6), 3.96 (1H, dd, J=1.8, 11.5 Hz, H-6), 4.01 (1H, d, J=1.8 Hz, H-11b), 6.32 (1H, d, J=2.2 Hz, H-4), 6.50 (1H, dd, J=2.2, 8.2 Hz, H-2), 6.65 (1H, s, H-11), 6.79 (1H, s, H-8), 7.21 (1H, d, J=8.2 Hz, H-1).

Protosappanin A (13)[9a]——Colorless needles, mp 254—257 °C (lit.[9a] 250—251 °C). UV λ_{max}^{MeOH} nm (log ε): 284 (3.58), 260 (4.04). EI-MS m/z: 272.0680 (M^+, Calcd for $C_{15}H_{12}O_5$: 272.0682), 244, 229. ^{1}H-NMR (acetone-d_6) δ: 3.44 (2H, s, H-8), 4.49 (2H, s, H-6), 6.65—6.90 (4H, m, H-2, 4, 9 and 12), 7.16 (1H, d, J=9.0 Hz, H-1).

Protosappanin B (14)[9b]——$[\alpha]_D^{25}$ −12.5° (c=0.64, MeOH). UV λ_{max}^{MeOH} nm (log ε): 288 (4.09), 254 (4.33). EI-MS m/z: 304 (M^+), 243, 231, 229, 228, 227, 213, 115. ^{1}H-NMR (acetone-d_6) δ: 2.60 (2H, H-8), 3.60 (2H, br s, 7-CH_2OH), 3.70—4.60 (2H, H-6), 6.54 (1H, H-4), 6.60 (1H, dd, J=2.0, 8.0 Hz, H-2), 6.67 (2H, H-9 and 12), 7.01 (1H, d, J=8.0 Hz, H-1).

Pentaacetate of 14[9b]——A mixture of **14** (264 mg), Ac_2O (88 mg) and Et_3N (120 mg) in dry benzene (30 ml) was stirred at room temperature for 18 h in the presence of a catalytic amount of 4-dimethylaminopyridine. The solution was worked up in a usual manner to afford the pentaacetate (368 mg). $[\alpha]_D^{25}$ −65.8° (c=3.20, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε): 280 (3.80), 245 (4.33). EI-MS m/z: 514 (M^+), 472, 430, 388, 346, 304. ^{1}H-NMR ($CDCl_3$) δ: 2.02, 2.09 and 2.30 (3H, 3H and 9H, each s, acetyl methyl), 2.71 (1H, br d, J=14.0 Hz, H-8), 3.50 (1H, br d, J=14.0 Hz, H-8), 4.00 (1H, br d, J=13.0 Hz, H-6), 4.44 and 4.54 (each 1H, br d, J=9.0 Hz, 7-CH_2OAc), 4.85 (1H, br d, J=13.0 Hz, H-6), 6.86 (1H, d, J=2.0 Hz, H-4), 6.90 (1H, dd, J=2.0, 8.0 Hz, H-2), 7.17 (1H, d, J=8.0 Hz, H-1), 7.20 (2H, s, H-9 and 12).

Protosappanin C (15)[9c]——$[\alpha]_D^{25}$ −37.0° (c=5.32, MeOH). UV λ_{max}^{MeOH} nm (log ε): 288 (3.75), 254 (3.97). EI-MS m/z: 302.0786 (M^+, Calcd for $C_{16}H_{14}O_6$: 302.0788), 230, 229, 213. ^{1}H-NMR (acetone-d_6) δ: 2.68—3.00 (2H, H-8), 3.90—4.50 (2H, H-6), 6.59 (1H, br s, H-4), 6.64 (1H, dd, J=2.0, 8.0 Hz, H-2), 6.82 (2H, H-9 and 12), 7.05 (1H, d, J=8.0 Hz, H-1), 9.68 (1H, s, 7-CHO).

$NaBH_4$ Treatment of 15——Sodium borohydride (120 mg) was added to a solution of **15** (146 mg) in MeOH

(10 ml) under stirring at 0 °C. The mixture was stirred at room temperature for 3 h, 1 N HCl (4 ml) and H_2O (30 ml) were added, and the MeOH was evaporated off. The residual H_2O solution was extracted with EtOAc (3 × 30 ml), and the EtOAc extract was washed with H_2O (3 × 30 ml) and sat. NaCl aq. (30 ml) successively, and dried over Na_2SO_4. The product obtained after evaporation of the solvent was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (10:2:0.1) to yield **14** (78 mg). The physicochemical properties of this compound coincided with those of protosappanin B.

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Synthesis of Peptide Chloromethyl Ketones and Examination of Their Inhibitory Effects on Human Spleen Fibrinolytic Proteinase (SFP) and Human Leukocyte Elastase (LE)[1]

YUKO TSUDA,[a] YOSHIO OKADA,*[,a] YOKO NAGAMATSU[b] and UTAKO OKAMOTO[b]

Faculty of Pharmaceutical Sciences[a] and Faculty of Nutrition,[b] Kobe-Gakuin University, Nishi-ku, Kobe 673, Japan

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Various substrate-derived chloromethyl ketones were synthesized by a conventional method for the purpose of obtaining specific and potent irreversible inhibitors for human spleen fibrinolytic proteinase (SFP) and human leukocyte elastase (LE) in order to compare the properties of SFP with those of LE. It was found that Boc–Ala–Tyr–Leu–Val–CH_2Cl among the peptide chloromethyl ketones exhibited the most effective and specific inhibition of SFP and LE. The two enzymes were inhibited by peptide chloromethyl ketones having a Val residue at the C-terminus in a similar manner, demonstrating a similarity between SFP and LE.

Keywords——human spleen fibrinolytic proteinase; human leukocyte elastase; peptide chloromethyl ketone; chemical synthesis; specific inhibition

Human spleen fibrinolytic proteinase (SFP)[2] and human leukocyte elastase (LE) have recently attracted our interest as non-plasmin fibrinolytic proteinases. The latter enzyme is responsible for the tissue destruction that occurs in pulmonary emphysema[3] and in inflammation.[4] Previously, we reported that Suc–Tyr–Leu–Val–*p*NA and Suc–Ala–Tyr–Leu–Val–*p*NA were specific substrates for SFP (k_{cat}/K_m=22600 and 84000 $M^{-1}s^{-1}$, respectively)[5-7] as well as LE (k_{cat}/K_m=17600 and 48500 $M^{-1}s^{-1}$, respectively). It was also indicated that the substrate specificities of these enzymes are similar. In addition, it was shown that stereoisomers of Suc–Tyr–Leu–Val–*p*NA, except for Suc–D-Tyr–Leu–Val–*p*NA, exhibited reversible inhibitory activity on both enzymes in a similar manner. In order to clarify further the enzymatic properties and physiological roles of SFP and LE, more potent and selective inhibitors were required.

Specific inhibitors are useful tools in understanding the properties and physiological roles of enzymes. For example, diisopropylphosphofluoridate (DIPF)[8] reacts stoichiometrically with the active site serine residue of serine proteases, thus making it useful in the initial characterization of an enzyme as a serine protease. On the other hand, Tos–Phe–CH_2Cl and Tos–Lys–CH_2Cl, developed by Shaw and his coworkers,[9] have proved to be inhibitors of chymotrypsin and trypsin, respectively. Substrate-derived chloromethyl ketones appeared to be candidates for use as potent and selective inhibitors against the corresponding enzymes.

In order to obtain effective and specific inhibitors against SFP and LE, we planned the synthesis of peptides having valine chloromethyl ketones at the C-terminus, because our previous studies had shown that SFP and LE cleaved valyl bonds most rapidly and specifically.[7] First of all, we designed and synthesized Boc–Tyr–Leu–Val–CH_2Cl [I], Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and peptide chloromethyl ketones [III—VI] with substitution of Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] at the P_3 or P_5 position. Next, we prepared 8 kinds of stereoisomeric Boc–Tyr–Leu–Val–CH_2Cl [I, VII—XIII] on the basis of our previous

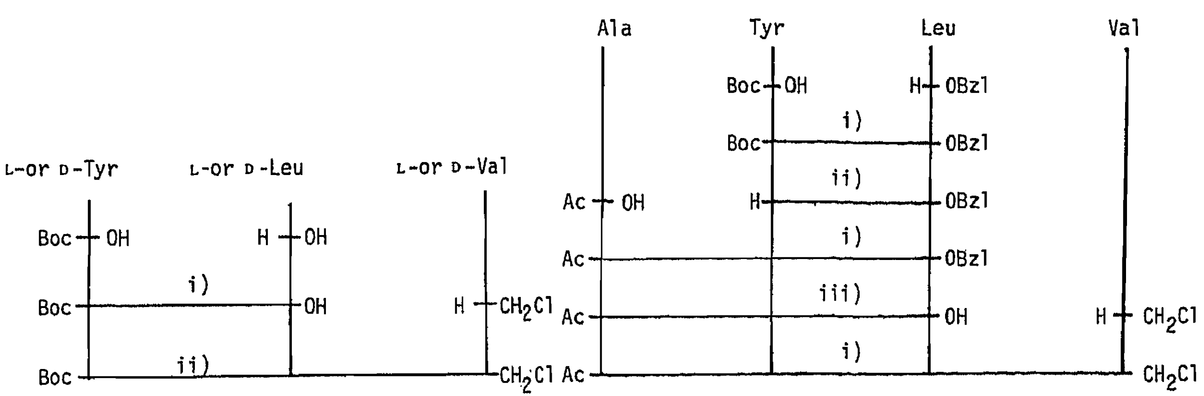

Fig. 1a. Synthetic Scheme for Tripeptide Chloromethyl Ketones

i) azide method; ii) DCC–HOBt method.

Fig. 1b. Synthetic Scheme for Tetrapeptide Chloromethyl Ketones

i) DCC–HOBt method; ii) HCl/dioxane; iii) H_2–Pd.

studies.[10] This paper deals with the synthesis of the series of peptide chloromethyl ketones, as well as their inhibitory effects on SFP in comparison with those on LE.

In earlier studies,[11-14] amino acid chloromethyl ketone was coupled with various kinds of N-protected peptides by the mixed anhydride method[15] to produce the corresponding peptide chloromethyl ketones. With regard to fragment condensation, the azide method[16] or the DCC–HOBt method[17] is generally employed in order to minimize racemization and avoid formation of the urethan-type derivative which was reported to occur during the coupling reaction between the mixed anhydride formed and the amino group of a bulky amino acid such as valine.[18] Thus, we attempted to prepare peptides having Val chloromethyl ketone at the C-terminus by the azide method. However, the resultant products were a mixture of the desired product and the urea derivative formed through Curtius rearrangement[19] judging from elemental analysis (data not shown). Therefore, the DCC–HOBt method was used to prepare peptides having valine chloromethyl ketone at the C-terminus, and purification by silica gel column chromatography was carried out, if necessary. As a typical experiment, two synthetic routes to peptide chloromethyl ketones are shown in Fig. 1a, b. The homogeneity of peptide chloromethyl ketones obtained was ascertained by thin-layer chromatography (TLC) on silica gel and amino acid and elemental analysis. The results are summarized in the experimental section.

The inhibitory effect of synthetic peptides was assayed by measuring the *p*-nitroaniline (E_{410}) released by the enzyme in the presence of the inhibitor. The kinetics of inhibition of SFP or LE by the peptide chloromethyl ketones were determined at three or four different concentrations. It appears that during the period employed, the reaction follows first-order kinetics, as shown in Fig. 2. From the slope of the curve, the inactivation rate constant (k) was calculated by using the equation, $k=0.693/T_{1/2}$ (where $T_{1/2}$ is the apparent half life in seconds) according to Ardelt *et al.*[20] Since the k values thus obtained approximate to k_{obsd},[11-13] we employed these k values described above as k_{obsd}.

The inhibitory effects of Boc–Tyr–Leu–Val–CH_2Cl [I], Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and its analogs [III—VI] modified at the P_3 or P_5 position of II on SFP and LE are summarized in Table I. Boc–Tyr–Leu–Val–CH_2Cl [I] was found to be a potent inhibitor of SFP and LE. Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] and its analogs [III—VI] are more reactive and selective than Boc–Tyr–Leu–Val–CH_2Cl, while the shorter analogs, Boc–Val–CH_2Cl and Boc–Leu–Val–CH_2Cl, are incapable of inhibiting SFP and LE under the same conditions. From these results, it can be deduced that the inhibitory effect of peptide chloromethyl ketones on SFP and LE is strongly influenced by the peptide chain length, as shown in the case of substrates.[5]

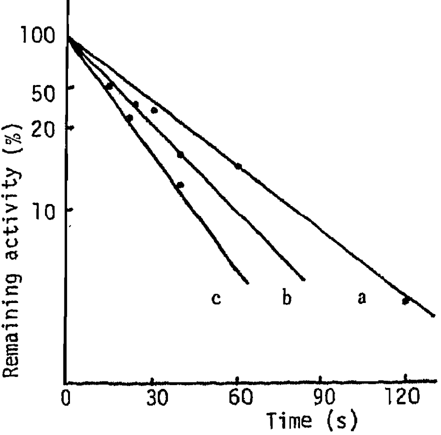

Fig. 2. Kinetics of Inactivation of SFP by Boc–Ala–Tyr–Leu–Val–CH_2Cl

The final concentrations were as follows: dioxane, 0.6% (v/v); SFP, 0.35 μM; inhibitor, 0.01 mM (a) or 0.015 mM (b) or 0.02 mM (c).

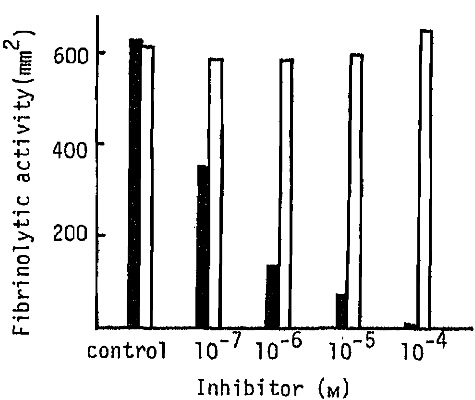

Fig. 3. Inhibitory Effects of Boc–Ala–Tyr–Leu–Val–CH_2Cl on the Fibrinolytic Activities of LE and PPE

■, LE; □, PPE (porcine pancreatic elastase). Boc–Ala–Tyr–Leu–Val–CH_2Cl was mixed with a fibrinogen solution before thrombin was added. The final concentrations of the inhibitor in the plates were 10^{-7} to 10^{-4} M.

TABLE I. Inhibitory Effects of Peptide Chloromethyl Ketones on the Amidolytic Activities of SFP and LE

Inhibitor	Enzyme	[I] (M) × 10^4	$10^4 \times k_{obsd}$ (s^{-1})	Half life (s)	k_{obsd}/[I] ($M^{-1}s^{-1}$)	k_{obsd}/[I] (rel.)
Boc–Val–CH_2Cl	SFP	0.25[a]	—	—	—	—
	LE	0.25[a]	—	—	—	—
Boc–Leu–Val–CH_2Cl	SFP	0.25[a]	—	—	—	—
	LE	0.25[a]	—	—	—	—
Boc–Tyr–Leu–Val–CH_2Cl [I]	SFP	0.1	224	31	2240	1.0
	LE	0.1	117	59	1170	0.52
Boc–Ala–Tyr–Leu–Val–CH_2Cl [II]	SFP	0.1	495	14	4950	2.2
	LE	0.1	330	21	3300	1.5
Boc–Ala–Phe–Leu–Val–CH_2Cl [III]	SFP			N.D.[b]		
	LE	0.05	165	42	3300	1.5
Ac–Ala–Tyr–Leu–Val–CH_2Cl [IV]	SFP	0.1	248	28	2480	1.1
	LE	0.1	151	46	1510	0.67
DNS–Ala–Tyr–Leu–Val–CH_2Cl [V]	SFP	0.1	301	23	3010	1.3
	LE	0.1	267	26	2670	1.2
DNS–Ala–Phe–Leu–Val–CH_2Cl [VI][c]						

The final concentrations were as follows: dioxane, 1.5% (v/v); enzyme, 0.35 μM, inhibitor, 0.1 or 0.05 mM. *a*) This compound did not show any inhibitory effect on the enzyme after preincubation for more than 10 min at this concentration. *b*) Not determined. *c*) The inhibitory activity was not determined, because this compound was not sufficiently soluble to give 0.1 mM concentration.

In order to determine the specificity of Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] against SFP and LE, we measured its inhibitory effect on fibrinolysis by porcine pancreatic elastase (PPE), plasmin, trypsin and α-chymotrypsin. As an example, the results for PPE are shown in Fig. 3. As expected, Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] did not inhibit PPE, plasmin, trypsin or α-chymotrypsin, indicating that Boc–Ala–Tyr–Leu–Val–CH_2Cl [II] is a specific inhibitor of SFP and LE.

Next, the inhibitory effects of 8 kinds of stereoisomers [I, VII—XIII] of Boc–Tyr–Leu–

Table II. Inhibitory Effects of Stereoisomers of Boc–Tyr–Leu–Val–CH_2Cl on the Amidolytic Activity of SFP and LE

Inhibitor Boc–Tyr–Leu–Val–CH_2Cl	[I] (mM)	SFP	LE
		Inhibition (%)	
L–L–L [I]	0.0025	40	20
	0.005	80	55
	0.05	100	100
D–L–L [VII]	0.05	60	30
D–D–L [VIII]	0.05	10	8
L–D–L [IX]	0.05	<5	<5
L–L–D [X]	0.05	10	5
D–L–D [XI]	0.05	10	5
D–D–D [XII]	0.05	0	0
L–D–D [XIII]	0.05	0	0

The preincubation was performed at 37 °C for 2 min. The final concentrations were as follows: dioxane, 1.5% (v/v); enzyme, 0.35 μM.

Val–CH_2Cl on the amidolytic activity of SFP and LE are summarized in Table II. From the results, it can be seen that SFP and LE are inhibited in a similar manner by those peptide chloromethyl ketones, demonstrating a further similarity between SFP and LE. In addition, the potency of the inhibitory activity of peptide chloromethyl ketones on SFP and LE is in inverse proportion to that of the corresponding *p*NA derivatives. Although it is reasonable that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not inhibit the enzymes because the chloromethyl ketone functional group might be facing away from the active site histidine residue, it is interesting that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not inhibit either enzyme, while Suc–Tyr–D-Leu–D-Val–*p*NA inhibited the amidolytic activity of SFP and LE toward Suc–Tyr–Leu–Val–*p*NA. The fact that Boc–Tyr–D-Leu–D-Val–CH_2Cl [XIII] did not show any inhibitory effect on SFP or LE supports our previous hypothesis[21] that the *p*NA moiety in Suc–Tyr–D-Leu–D-Val–*p*NA is required to interact with some part of the enzymes for manifestation of inhibitory activity.

In conclusion, the results presented in this paper demonstrate that peptide chloromethyl ketones having a Val residue at the P_1 position are potent and specific inhibitors of SFP and LE. The similar modes of action of SFP and LE toward those peptide chloromethyl ketones suggest that the two enzymes have quite similar three-dementional structures around the active center. These substrate-derived chloromethyl ketones should be useful tools for the clarification of the roles of SFP and LE and for distinguishing these enzymes from other enzymes in the complex physiological environment.

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 (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer (K-101AS, Kyowa Seimitsu). For column chromatography, a Toyo SF-160K fraction collector was used. For TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), $CHCl_3$, MeOH and H_2O (89 : 10 : 1), $CHCl_3$, MeOH and H_2O (97 : 2.8 : 0.2), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase), *n*-BuOH, AcOH, pyridine and H_2O (4 : 1 : 1 : 2), respectively. Mass spectra (MS) were measured with a Hitachi RMU-7MG mass spectrometer by the field desorption (FD) technique.

Z–Val–CH_2Cl——Diazomethane [prepared from nitrosomethylurea (6.1 g, 60 mmol)] was added to a mixed anhydride [prepared from Z–Val–OH (7.5 g, 30 mmol), Et_3N (4.2 ml, 30 mmol) and ethyl chloroformate (2.8 ml,

30 mmol)] in THF (100 ml) at $-15\,°C$ and the reaction mixture was stirred for 15 h at 4 °C. After addition of 8.4 N HCl/dioxane (8.0 ml, 67 mmol) at $-15\,°C$, the reaction mixture was stirred for 3 h at $-15\,°C$. After neutralization of the solution with Et_3N and removal of the solvent, the residue was dissolved in AcOEt. This solution was washed with 0.1 N HCl, 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a crystalline meterial, which was recrystallized from EtOH, yield 6.2 g (73%), mp 69—74 °C, $[\alpha]_D^{25}$ $-24.3°$ (c=1.0, MeOH), Rf^1 0.73. *Anal.* Calcd for $C_{14}H_{18}ClNO_3$: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.6; H, 6.49; N, 4.83.

Z-D-Val-CH$_2$Cl——The title compound was prepared from Z-D-Val-OH (7.5 g) in the same manner as described above, yield 5.0 g (59%), mp 74—76 °C, $[\alpha]_D^{25}$ $+21.7°$ (c=1.0, MeOH), Rf^1 0.73. *Anal.* Calcd for $C_{14}H_{18}ClNO_3$: C, 59.3; H, 6.39; N, 4.93. Found: C, 59.4; H, 6.41; N, 4.98.

Boc-Val-CH$_2$Cl——The title compound was prepared from Boc-Val-OH (6.3 g) in the same manner as described above, yield 4.8 g (64%), mp 68—69 °C, $[\alpha]_D^{25}$ $-34.4°$ (c=1.0, MeOH), Rf^1 0.81. *Anal.* Calcd for $C_{11}H_{20}ClNO_3$: C, 52.9; H, 8.07; N, 5.60. Found: C, 52.6; H, 8.16; N, 5.65.

Boc-D-Val-CH$_2$Cl——The title compound was prepared from Boc-D-Val-OH (6.3 g) in the same manner as described above, yield 2.4 g (32%), mp 67—69 °C, $[\alpha]_D^{25}$ $+33.9°$ (c=1.0, MeOH), Rf^1=0.81. *Anal.* Calcd for $C_{11}H_{20}ClNO_3$: C, 52.9; H, 8.07; N, 5.60. Found: C, 52.7; H, 8.21; N, 5.78.

Boc-Leu-Val-CH$_2$Cl——Boc-Leu-OH (2.5 g, 0.01 mol), HOBt (1.4 g, 0.01 mol) and H-Val-CH$_2$Cl·HBr [prepared from Z-Val-CH$_2$Cl (2.8 g, 0.01 mol) and 25% HBr/AcOH (9.7 ml, 0.03 mol)] were dissolved in DMF (20 ml) containing Et_3N (1.4 ml). DCC (2.3 g, 0.011 mol) was added to the above cold solution and the reaction mixture was stirred for 1 h at $-15\,°C$ and for 18 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give an oily material. The crude product in $CHCl_3$ was applied to a silica gel column (1.5×20 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (400—500 ml) was removed by evaporation. Petroleum ether was added to the residue to provide the purified material, yield 0.59 g (17%), mp 159—161.5 °C, $[\alpha]_D^{25}$ $-54.6°$ (c=1.1, MeOH), Rf^1 0.81, Rf^3 0.79. *Anal.* Calcd for $C_{17}H_{31}ClN_2O_3 \cdot H_2O$: C, 56.0; H, 9.11; N, 7.67. Found: C, 56.4; H, 8.90; N, 7.87.

General Procedure for the Synthesis of Stereoisomeric Boc-Tyr-Leu-OH——Boc-Tyr-N$_3$ [prepared from Boc-Tyr-N$_2$H$_3$ (2.0 g, 6.8 mmol), 8.4 N HCl/dioxane (1.6 ml, 14 mmol) and isoamyl nitrite (0.94 ml, 6.8 mmol) at $-40\,°C$] in DMF (10 ml) were added to a solution of H-Leu-OH (0.88 g, 6.8 mmol) in H_2O (20 ml) and DMF (10 ml) containing Et_3N (0.95 ml, 6.8 mmol). The reaction mixture was stirred for 30 min at $-15\,°C$ and for 48 h at 4 °C. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ and this solution was washed with AcOEt. The aqueous layer was acidified with citric acid and the resultant oily material was extracted with AcOEt and washed with H_2O. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Petroleum ether was added to the residue to give an amorphous powder. The yield, melting point, $[\alpha]_D$ value, Rf values and analytical data are summarized in Table III.

General Procedures for the Synthesis of Stereoisomeric Boc-Tyr-Leu-Val-CH$_2$Cl [I, VII—XIII]——Boc-Tyr-Leu-OH (0.39 g, 1.0 mmol), HOBt (0.13 g, 1.0 mmol) and H-Val-CH$_2$Cl·HBr [prepared from Z-Val-CH$_2$Cl (0.28 g, 1.0 mmol) and 25% HBr/AcOH (1.0 ml, 3.0 mmol)] were dissolved in DMF (10 ml) containing Et_3N (0.14 ml). DCC (0.25 g, 1.2 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at $-15\,°C$ and for 18 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (1.5×35.5 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (500—700 ml) was removed by evaporation. Ether was added to the residue to provide the purified material.

TABLE III. Yield, Melting Point, Optical Rotation, Rf Values and Analytical Data of Boc-Tyr-Leu-OH

Compound Boc-Tyr-Leu-OH	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis			TLC	
					C	H	N	Rf^1	Rf^2
L-L	37	Amorphous	-5.8 (c=1.0)	$C_{20}H_{30}N_2O_6$	60.9 (61.0	7.66 7.87	7.10 6.96)	0.17	0.26
L-D	60	Amorphous	$+12.6$ (c=0.5)	$C_{20}H_{30}N_2O_6$	60.9 (60.9	7.66 7.86	7.10 6.99)	0.18	0.25
D-L	56	Amorphous	-13.5 (c=0.9)	$C_{20}H_{30}N_2O_6$	60.9 (60.9	7.66 7.93	7.10 6.87)	0.19	0.27
D-D	56	Amorphous	$+6.3$ (c=1.0)	$C_{20}H_{30}N_2O_6$	60.9 (60.8	7.66 7.82	7.10 6.94)	0.16	0.26

TABLE IV. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Boc–Tyr–Leu–Val–CH_2Cl

Compound Boc–Tyr–Leu–Val–CH_2Cl	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (MeOH)	Formula	Elemental analysis			TLC	
					C	H	N	Rf^1	Rf^2
L–L–L	9.4	125—127	−42.5 (c=0.4)	$C_{26}H_{40}ClN_3O_6 \cdot 1/2H_2O$	58.4 (58.5	7.72 7.63	7.85 7.90)	0.40	0.70
D–L–L	12	169—170	−80.6 (c=0.3)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.7	7.66 7.98	7.98 8.33)	0.41	0.69
D–D–L	13	88—89	−18.0 (c=0.1)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.5	7.66 7.87	7.98 7.84)	0.43	0.66
L–D–L	8.6	80—87	+5.5 (c=0.4)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.9	7.66 8.04	7.98 7.61)	0.44	0.67
L–L–D	28	89—95	+18.1 (c=0.5)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.6	7.66 7.91	7.98 7.75)	0.45	0.68
D–L–D	14	74—80	−6.8 (c=0.6)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.5	7.66 7.87	7.98 7.74)	0.45	0.69
D–D–D	10	119—121	+39.4 (c=0.7)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.1	7.66 7.79	7.98 7.69)	0.48	0.69
L–D–D	6.1	169—170	+76.0 (c=0.4)	$C_{26}H_{40}ClN_3O_6$	59.4 (59.4	7.66 7.83	7.98 7.98)	0.48	0.70

The yield, melting point, $[\alpha]_D$ value, *Rf* values and analytical data are summarized in Table IV.

Boc–Ala–Tyr–N_2H_3——H–Tyr–OMe·HCl (6.9 g, 30 mmol), Boc–Ala–OH (5.6 g, 30 mmol) and HOBt (4.0 g, 30 mmol) were dissolved in DMF (30 ml) containing Et_3N (4.2 ml). DCC (8.2 g, 40 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at room temperature. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a white precipitate, which was collected by filtration. The crude Boc–Ala–Tyr–OMe was dissolved in MeOH (30 ml), and 80% hydrazine hydrate (4.6 ml, 74 mmol) was added. The reaction mixture was allowed to stand for 15 h at room temperature. The resultant precipitate was collected by filtration, and recrystallized from MeOH, yield 4.4 g (40%), mp 184—186 °C, $[\alpha]_D^{25}$ −16.0° (c=1.0, AcOH), Rf^1 0.20. *Anal.* Calcd for $C_{17}H_{26}N_4O_5$: C, 55.7; H, 7.15; N, 15.3. Found: C, 55.5; H, 7.14; N, 15.2.

Boc–Ala–Tyr–Leu–OH——Boc–Ala–Tyr–N_3 [prepared from Boc–Ala–Tyr–N_2H_3 (3.6 g, 10 mmol), 8.4 N HCl/dioxane (2.4 ml, 20 mmol) and isoamyl nitrite (1.4 ml, 10 mmol) at −40 °C] in DMF (30 ml) was added to a solution of H–Leu–OH (1.4 g, 10 mmol) in H_2O (60 ml) and DMF (30 ml) containing Et_3N (1.4 ml, 10 mmol). The reaction mixture was stirred for 30 min at −40 °C and for 48 h at 4 °C. After removal of the solvent by evaporation, the residue was dissolved in 5% $NaHCO_3$ and this solution was washed with AcOEt. The aqueous layer was acidified with citric acid and the resultant oily material was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated under reduced pressure. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2.5 × 33 cm) equilibrated with $CHCl_3$, and eluted with $CHCl_3$ (600 ml), 1% MeOH/$CHCl_3$ (600 ml) and then 2% MeOH/$CHCl_3$ (2000 ml). The solvent of the 2% MeOH/$CHCl_3$ effluent (700—2000 ml) was removed by evaporation. Ether was added to the residue to give a white powder, yield 1.8 g (39%), mp 113—115 °C, $[\alpha]_D^{25}$ −29.5° (c=1.0, MeOH), Rf^1 0.20. *Anal.* Calcd for $C_{23}H_{35}N_3O_7$: C, 59.3; H, 7.57; N, 9.02. Found: C, 59.7; H, 7.89; N, 8.57.

Boc–Ala–Tyr–Leu–Val–CH_2Cl [II]——Boc–Ala–Tyr–Leu–OH (0.54 g, 1.2 mmol), HOBt (0.16 g, 1.2 mmol) and H–Val–CH_2Cl·HCl [prepared from Boc–Val–CH_2Cl (0.25 g, 1.0 mmol) and 2.7 N HCl/dioxane (1.8 ml, 5.0 mmol)] were dissolved in DMF (10 ml) containing Et_3N (0.14 ml). DCC (0.30 g, 1.5 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 48 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether and ether were added to the residue to give a light yellow precipitate, which was recrystallized from $CHCl_3$–ether, yield 0.10 g (17%), mp 172—175 °C, $[\alpha]_D^{25}$ −63.1° (c=0.3, MeOH), Rf^1 0.29. *Anal.* Calcd for $C_{29}H_{45}ClN_4O_7$: C, 58.3; H, 7.60; N, 9.38. Found: C, 58.2; H, 7.57; N, 9.47. FD-MS *m/z*: 597 (M^+).

Boc–Ala–Phe–Leu–Val–CH_2Cl [III]——The title compound was prepared from Boc–Ala–Phe–Leu–OH[22,23] (0.90 g) and H–Val–CH_2Cl [prepared from Z–Val–CH_2Cl (0.70 g)] in the same manner as described for the preparation of II, yield 0.36 g (31%), mp 114—118 °C, $[\alpha]_D^{25}$ −59.6° (c=1.0, MeOH), Rf^1 0.56, Rf^2 0.85. *Anal.* Calcd

for $C_{29}H_{45}ClN_4O_6$: C, 59.9; H, 7.80; N, 9.64. Found: C, 59.7; H, 7.89; N, 9.43.

Boc–Tyr–Leu–OBzl——Boc–Tyr–OH (2.8 g, 10 mmol), H–Leu–OBzl·TosOH (3.9 g, 10 mmol) and HOBt (1.3 g, 10 mmol) were dissolved in DMF (50 ml) containing Et_3N (1.4 ml, 10 mmol). DCC (2.3 g, 11 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2.5 × 30 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (750—1200 ml) was removed by evaporation. Petroleum ether was added to the residue to give an amorphous powder, yield 2.3 g (49%), $[\alpha]_D^{25}$ −18.2° (*c*=1.0, MeOH), *Rf*¹ 0.68, *Rf*² 0.64. *Anal.* Calcd for $C_{27}H_{36}N_2O_6$: C, 66.9; H, 7.49; N, 5.79. Found: C, 66.6; H, 7.53; N, 6.07.

Ac–Ala–Tyr–Leu–OBzl——Ac–Ala–OH (0.20 g, 1.6 mmol), H–Tyr–Leu–OBzl·HCl [prepared from Boc–Tyr–Leu–OBzl (0.94 g, 2.0 mmol) and 3.6 N HCl/dioxane (2.8 ml, 10 mmol)] and HOBt (0.22 g, 1.6 mmol) were dissolved in DMF (10 ml) containing Et_3N (0.28 ml). DCC (0.41 g, 2.0 mmol) was added to the above cold solution and the reaction mixture was stirred for 18 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was recrystallized from AcOEt, yield 0.61 g (61%), mp 140—144 °C, $[\alpha]_D^{25}$ −47.4° (*c*=1.0, MeOH), *Rf*¹ 0.22, *Rf*³ 0.20. *Anal.* Calcd for $C_{27}H_{35}N_3O_6$: C, 65.2; H, 7.09; N, 8.45. Found: C, 65.1; H, 7.19; N, 8.66.

Ac–Ala–Tyr–Leu–OH——Ac–Ala–Tyr–Leu–OBzl (0.56 g, 1.1 mmol) was dissolved in MeOH (50 ml) and hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the oily residue to give a precipitate, which was collected by filtration, yield 0.35 g (78%), mp 224—229 °C, $[\alpha]_D^{25}$ −44.3° (*c*=0.9, MeOH), *Rf*¹ 0.10, *Rf*⁵ 0.69, *Rf*⁶ 0.63. *Anal.* Calcd for $C_{20}H_{29}N_3O_6$: C, 59.0; H, 7.17; N, 10.3. Found: C, 59.0; H, 7.36; N, 9.97.

Ac–Ala–Tyr–Leu–Val–CH₂Cl [IV]——Ac–Ala–Tyr–Leu–OH (0.20 g, 0.50 mmol), H–Val–CH_2Cl·HBr [prepared from Z–Val–CH_2Cl (0.18 g, 0.63 mmol) and 25% HBr/AcOH (0.60 ml, 1.9 mmol)] and HOBt (0.070 g, 0.50 mmol) were dissolved in DMF (40 ml) containing Et_3N (0.088 ml). DCC (0.12 g, 0.60 mmol) was added to the above cold solution and the reaction mixture was stirred for 1 h at −15 °C and for 18 h at 4 °C. After removal of the dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2.0 × 31 cm) equilibrated with $CHCl_3$. After elution with $CHCl_3$ (900 ml) and 3% MeOH/$CHCl_3$ (900 ml), the solvent of the latter effluent (300—900 ml) was removed by evaporation. Ether was added to the residue to give a white powder, yield 0.040 g (12%), mp 183—185 °C, $[\alpha]_D^{25}$ −40.2° (*c*=0.2, MeOH), *Rf*¹ 0.53, *Rf*³ 0.42. *Anal.* Calcd for $C_{26}H_{39}ClN_4O_6\cdot 1/4H_2O$: C, 57.5; H, 7.32; N, 10.3. Found: C, 57.3; H, 7.39; N, 10.4.

DNS–Ala–Tyr–Leu–OBzl——The title compound was prepared from DNS–Ala–OH (0.80 g) and H–Tyr–Leu–OBzl [prepared from Boc–Tyr–Leu–OBzl (1.2 g)] in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OBzl, yield 1.1 g (64%), mp 125—130 °C, $[\alpha]_D^{25}$ −37.5° (*c*=0.9, MeOH), *Rf*¹ 0.64, *Rf*³ 0.70. *Anal.* Calcd for $C_{37}H_{44}N_4O_7S$: C, 64.5; H, 6.43; N, 8.13. Found: C, 64.4; H, 6.48; N, 7.90.

DNS–Ala–Tyr–Leu–OH——The title compound was prepared from DNS–Ala–Tyr–Leu–OBzl (1.0 g) in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OH, yield 0.72 g (80%), mp 220—222.5 °C, $[\alpha]_D^{25}$ −37.6° (*c*=1.0, DMF), *Rf*¹ 0.32, *Rf*³ 0.08. *Anal.* Calcd for $C_{30}H_{38}N_4O_7S$: C, 60.2; H, 6.39; N, 9.35. Found: C, 60.0; H, 6.29; N, 9.06.

DNS–Ala–Tyr–Leu–Val–CH₂Cl [V]——The title compound was prepared from DNS–Ala–Tyr–Leu–OH (0.30 g) and H–Val–CH_2Cl [prepared from Z–Val–CH_2Cl (0.18 g)] in the same manner as described for the preparation of IV. The crude material in $CHCl_3$ was applied to a silica gel column (2.0 × 29.5 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (500—800 ml) was removed by evaporation. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.076 g (17%), mp 203—206 °C, $[\alpha]_D^{25}$ −41.0° (*c*=1.0, DMF), *Rf*¹ 0.70, *Rf*³ 0.70. *Anal.* Calcd for $C_{36}H_{38}ClN_5O_7S$: C, 59.2; H, 6.62; N, 9.59. Found: C, 59.0; H, 6.80; N, 9.67.

Boc–Phe–Leu–OBzl——The title compound was prepared from Boc–Phe–OH (2.7 g) and H–Leu–OBzl·TosOH (3.9 g) in the same manner as described for the preparation of Boc–Tyr–Leu–OBzl, yield 1.7 g (36%), mp 87.5—89.5 °C, $[\alpha]_D^{25}$ −23.0° (*c*=0.9, MeOH), *Rf*¹ 0.84, *Rf*³ 0.72. *Anal.* Calcd for $C_{27}H_{36}N_2O_5$: C, 69.2; H, 7.74; N, 5.98. Found: C, 69.0; H, 7.81; N, 6.21.

Boc–Ala–Phe–Leu–OBzl——The title compound was prepared from Boc–Ala–OH (0.57 g) and H–Phe–Leu–OBzl [prepared from Boc–Phe–Leu–OBzl (1.4 g)] in the same manner as described for the preparation of Ac–Ala–Tyr–Leu–OBzl, yield 0.53 g (33%), amorphous powder, $[\alpha]_D^{25}$ −24.8° (*c*=1.0, DMF), *Rf*¹ 0.57, *Rf*³ 0.58. *Anal.* Calcd for $C_{30}H_{41}N_3O_6$: C, 66.8; H, 7.61; N, 7.79. Found: C, 66.8; H, 7.58; N, 7.93.

DNS–Ala–Phe–Leu–OBzl——DNS–Cl (0.72 g, 2.7 mmol) was dropped into a solution of H–Ala–Phe–Leu–OBzl·HCl [prepared from Boc–Ala–Phe–Val–OBzl (1.2 g, 2.2 mmol) and 3.7 N HCl/dioxane (6.0 ml, 22 mmol)] in DMF (20 ml) containing Et_3N (0.31 ml, 2.2 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and for 1 h

at room temperature. After removal of the solvent, AcOEt was added to the residue to give a precipitate, which was collected by filtration and recrystallized from EtOH, yield 1.5 g (100%), mp 224—226 °C, $[\alpha]_D^{25}$ −42.4° (c=1.0, DMF), Rf^1 0.56, Rf^2 0.87, Rf^4 0.40. *Anal.* Calcd for $C_{37}H_{44}N_4O_6S$: C, 66.0; H, 6.59; N, 8.32. Found: C, 66.0; H, 6.56; N, 8.55.

DNS–Ala–Phe–Leu–OH——The title compound was obtained from DNS–Ala–Phe–Leu–OBzl (1.3 g) by hydrogenation over a Pd catalyst in DMF (40 ml). After removal of Pd and the solvent, EtOH was added to the residue to give a yellow precipitate, which was collected by filtration, yield 1.1 g (95%), mp 216—220 °C, $[\alpha]_D^{25}$ −38.6° (c=1.0, DMF), Rf^1 0.38, Rf^2 0.50. *Anal.* Calcd for $C_{30}H_{38}N_4O_6S \cdot 1/2H_2O$: C, 60.9; H, 6.64; N, 9.46. Found: C, 60.4; H, 6.87; N, 10.0.

DNS–Ala–Phe–Leu–Val–CH_2Cl [VI]——A mixed anhydride [prepared from DNS–Ala–Phe–Leu–OH (1.0 g, 1.7 mmol), Et_3N (0.24 ml, 1.7 mmol) and ethyl chloroformate (0.24 ml, 1.7 mmol) at −15 °C] in DMF (10 ml) was added to a solution of H–Val–$CH_2Cl \cdot HBr$ [prepared from Z–Val–CH_2Cl (0.59 g, 2.1 mmol) and 25% HBr/AcOH (0.24 ml, 6.3 mmol)] in DMF (10 ml) containing Et_3N (0.24 ml, 1.7 mmol). The reaction mixture was stirred for 1 h at −15 °C and for 15 h at 4 °C. After removal of the solvent, the residue was dissolved in AcOEt and this solution was washed with 5% $NaHCO_3$, 0.1 N HCl and H_2O, dried over Na_2SO_4 and concentrated to a small volume. Petroleum ether was added to the residue to give a precipitate, which was collected by filtration. The crude material in $CHCl_3$ was applied to a silica gel column (2×27 cm) equilibrated and eluted with $CHCl_3$. The solvent of the effluent (700—1100 ml) was removed by evaporation. Ether was added to the residue to provide the purified material, yield 0.14 g (9.3%), mp 215—218 °C, $[\alpha]_D^{25}$ −48.2° (c=1.0, DMF), Rf^1 0.48, Rf^4 0.10. *Anal.* Calcd for $C_{36}H_{48}ClN_5O_6S$: C, 60.5; H, 6.84; N, 9.80. Found: C, 60.3; H, 6.70; N, 9.85.

Assay Procedure——SFP and LE were purified by gel-filtration[2] and affinity chromatography.[24] SFP and LE eluted with 8 M urea from the affinity column were used after dialysis against 0.1 M Tris–HCl buffer (pH 8.0) containing 2 M $NaClO_4$. All synthetic substrates and inhibitors were dissolved in 0.1 M Tris–HCl buffer (pH 8.0) containing dioxane. The final concentrations of dioxane, enzyme and inhibitor were as indicated in Fig. 2 and Tables I and II. The enzyme solution was mixed with an equal volume of the inhibitor at 37 °C, and preincubation was continued until the addition of an excess of the substrate. The remaining amidolytic activity of the enzyme was measured at intervals by using Suc–Ala–Tyr–Leu–Val–*p*NA[5] (0.5 mM) as the substrate, and the inhibitory activity was estimated by comparison with the amidolytic activity after a preincubation time of 0 second. The fibrinolytic activity was estimated with plasminogen-free fibrin plates in essentially the same manner as described previously.[24]

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References and Notes

1) All amino acid residues are of L-configuration unless otherwise indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, 5, 2485 (1966); *ibid.*, 6, 362 (1967); *ibid.*, 11, 1726 (1972). Other abbreviations used are: Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Suc, succinyl; Ac, acetyl; DNS, dansyl; *p*NA, *p*-nitroanilide; OBzl, benzyl ester; Et_3N, triethylamine; AcOH, acetic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; AcOEt, ethyl acetate; THF, tetrahydrofuran; *n*-BuOH, *n*-butanol.
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Studies on Peptides. CLIV.[1,2] Synthesis of a 36-Residue Peptide Amide Corresponding to the Entire Amino Acid Sequence of Human Pancreatic Polypeptide

NAOKI SUGIYAMA,[a] NOBUTAKA FUJII,[a] SUSUMU FUNAKOSHI,[a] AKIHIRO FUNAKOSHI,[b] KYOKO MIYASAKA,[c] MITSURU AONO,[d] MOTOYUKI MORIGA,[d] KAZUTOMO INOUE,[d] MASAFUMI KOGIRE,[d] SHOICHIRO SUMI,[d] RYUICHIRO DOI,[d] TAKAYOSHI TOBE,[d] and HARUAKI YAJIMA*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Kyoto 606, Japan, 3rd Department of Internal Medicine, Kyushu University,[b] Fukuoka 812, Japan, Tokyo Metropolitan Institute of Gerontology,[c] Itabashi-ku, Tokyo 173, Japan, and Faculty of Medicine, Kyoto University,[d] Kyoto 606, Japan

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A 36-residue peptide corresponding to the entire amino acid sequence of human pancreatic polypeptide (hPP) was synthesized by the solution method. A new Asp derivative, Asp(OMen) [Men = menthyl] was employed. Seven fragments served to construct the peptide backbone of hPP and all the protecting groups employed were cleaved by 2 M trimethylsilyl trifluoromethanesulfonate–diphenylsulfide/trifluoroacetic acid. The synthetic peptide inhibited protein secretion from rat pancreas.

Keywords——human pancreatic polypeptide synthesis; β-menthylaspartate; hard acid deprotection; trimethylsilyl trifluoromethanesulfonate; soft base; diphenylsulfide-mediated deprotection; inhibited pancreatic secretion

The structure of human pancreatic polypeptide (hPP), a 36-residue peptide amide, was proposed by Floyd *et al.*[3] in 1977 and its sequence homology to other mammalian PPs[3,4] and avian PP[5] was disclosed. However, two residues of hPP, Asp or Asn, at positions 10 and 11 have remained to be clarified. In 1984, Leiter *et al.*[6] and Boel *et al.*[7] deduced the complete amino acid sequence of prepro-hPP from the nucleotide sequence of complementary deoxyribonucleic acid (cDNA) encoding the precursor. This gene structure was compared with the nucleotide sequence of cDNA constructed with ribonucleic acid (RNA) from a pancreatic neoplasm by Takeuchi and Yamada[8] and with that of rat PP by Yamamoto *et al.*[9] Thus, the sequence of hPP predicted from the cDNAs was found to be identical to that determined by the protein sequencing method, and the Asp–Asn residues (positions 10—11) that had been uncertain were firmly confirmed. Before these structural confirmations, a solid-phase synthesis of the proposed sequence of hPP was reported by Meyer and Coy,[10] and a solution-phase synthesis was orally presented by Yanaihara *et al.*[11]

In this paper, we wish to report the unambiguous solution-phase synthesis of a 36-residue peptide amide corresponding to the firmly established amino acid sequence of hPP. Our newly introduced deprotecting procedure with TMSOTf/TFA[12] and a new Asp derivative, Asp(OMen),[13] were successfully employed for the present synthesis.

We selected seven fragments as building blocks to construct the entire peptide backbone of this pancreatic polypeptide (Fig. 1). The TFA-labile Boc or Z(OMe) group was used for temporary N^{α} protection and amino acid derivatives bearing protecting groups removable by

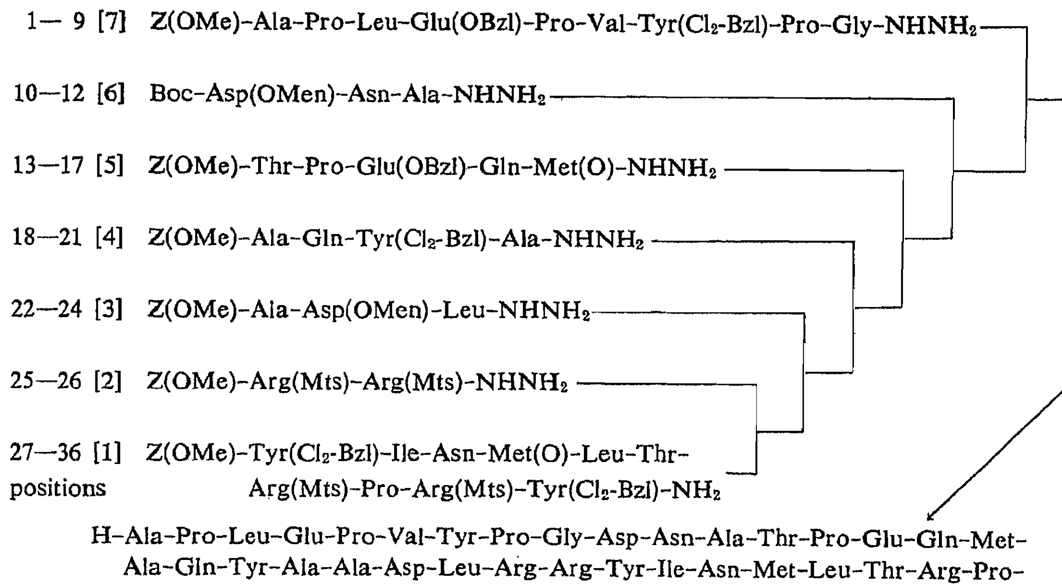

Fig. 1. Synthetic Route to Human Pancreatic Polypeptide

1 M TMSOTf–thioanisole/TFA were employed, *i.e.*, Tyr(Cl_2-Bzl)[14] Arg(Mts),[15] Glu(OBzl), and Asp(OMen). This peptide possesses four Tyr residues; one at the C-terminus and the others at positions 7, 20 and 27. In order to avoid over-acylation at the phenolic function, we selected Tyr(Cl_2-Bzl). This peptide possesses two Asp residues at positions 10 and 23. Of these, the former linked to the Asn residue is known to be particularly base-sensitive, forming succinimide.[16] Asp(OMen) was recently devised in order to suppress this base-catalyzed succinimide formation. Our model experiments showed that the Men group was cleaved by 1 M TMSOTf–diphenylsulfide/TFA more readily than 1 M TFMSA–thioanisole/TFA.[17] The Met residues at positions 17 and 30 were reversibly protected as the sulfoxide[18] to prevent air oxidation during manipulation and S-alkylation during N^{α}-deprotection. A substituted hydrazine, Troc–$NHNH_2$,[19] was employed to prepare fragments containing Glu(OBzl) and Asp(OMen) residues. Of the seven fragments, Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$ [2] is a known compound.[20]

The C-terminal decapeptide fragment [1], Z(OMe)–Tyr(Cl_2-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl_2-Bzl)–NH_2 (positions 27—36), was prepared by successive azide condensations[21] of three tripeptide units, followed by single addition of the Tyr(Cl_2-Bzl) residue as shown in Fig. 2. The C-terminal tripeptide unit, Boc–Pro–Arg-(Mts)–Tyr(Cl_2-Bzl)–NH_2, was prepared in a stepwise manner starting with a TFA-treated sample of Boc–Tyr(Cl_2-Bzl)–NH_2, to which Z(OMe)–Arg–(Mts)–OH and Boc–Pro–OH were successively condensed by the mixed anhydride (MA) method.[22] In order to prepare the middle unit, Z(OMe)–Leu–Thr–Arg(Mts)–$NHNH_2$, the known dipeptide, Z(OMe)–Leu–Thr–OMe,[23] was converted to the corresponding hydrazide, then condensed with H–Arg(Mts)–OMe, *via* the azide. The resulting protected tripeptide was treated with hydrazine hydrate as usual. The next unit, Z(OMe)–Ile–Asn–Met(O)–$NHNH_2$, was prepared in a stepwise manner by the active ester method. Z(OMe)–Asn–OH and Z(OMe)–Ile–OH were successively condensed, *via* the corresponding Np esters,[24] with a TFA-treated sample of Z(OMe)–Met(O)–OMe and the resulting protected tripeptide ester was converted to the corresponding hydrazide as stated above. Three peptide units thus obtained were condensed successively by the azide procedure, then the resulting nonapeptide chain was elongated by Su condensation[25] of Z(OMe)–Tyr(Cl_2-Bzl)–OH to give [1]. The purity of [1]

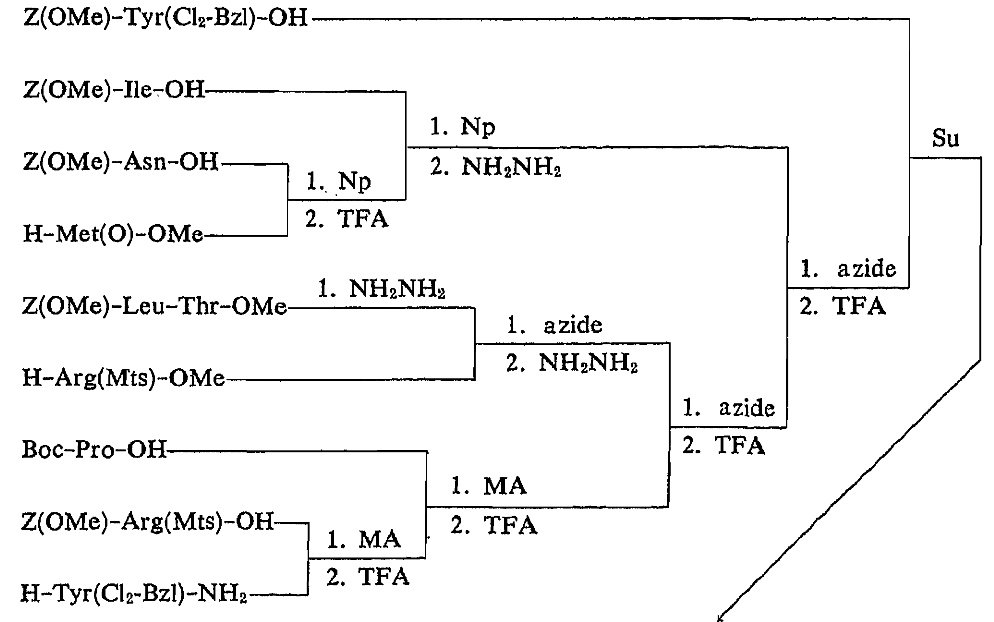

Fig. 2. Synthetic Scheme for the C-Terminal Protected Decapeptide Amide (27—36)

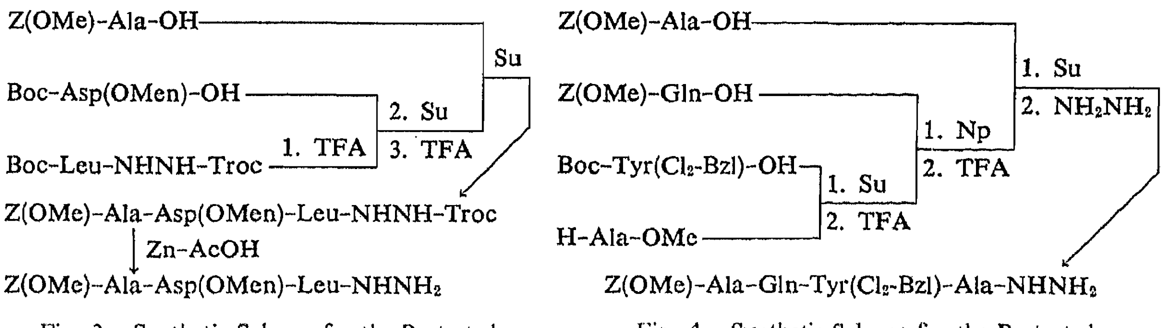

Fig. 3. Synthetic Scheme for the Protected Tripeptide Hydrazide (22—24)

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide (18—21)

was ascertained by thin layer chromatography (TLC), elemental analysis, and amino acid analysis after 6 N HCl hydrolysis, as was done with other fragments.

Fragment [3], Z(OMe)–Ala–Asp(OMen)–Leu–NHNH$_2$ (positions 22—24), was prepared with the aid of Troc–NHNH$_2$ as shown in Fig. 3. The Su active ester procedure was employed to condense successively Boc–Asp(OMen)–OH and Z(OMe)–Ala–OH onto a TFA-treated sample of Boc–Leu–NHNH–Troc. From the resulting protected tripeptide derivative, the Troc group was removed by treatment with Zn–AcOH[26] to give [3].

Fragment [4], Z(OMe)–Ala–Gln–Tyr(Cl$_2$-Bzl)–Ala–NHNH$_2$ (positions 18—21), was prepared in a stepwise manner by the active ester method as shown in Fig. 4. The Su method was employed to introduce two residues, Boc–Tyr(Cl$_2$-Bzl)–OH and Z(OMe)–Ala–OH, and the Np method for Z(OMe)–Gln–OH. The resulting protected tetrapeptide ester was less soluble in DMF. Thus, with the aid of HMPA as a solvent, it was converted to [4].

Next, the Glu(OBzl)-containing fragment [5], Z(OMe)–Thr–Pro–Glu(OBzl)–Gln–Met(O)–NHNH$_2$ (positions 13—17), was prepared with the aid of Troc–NHNH$_2$ as employed for fragment [3]. As shown in Fig. 5, the Np method was employed to condense successively Z(OMe)–Gln–OH and Z(OMe)–Glu(OBzl)–OH, then the DCC–HOSu pro-

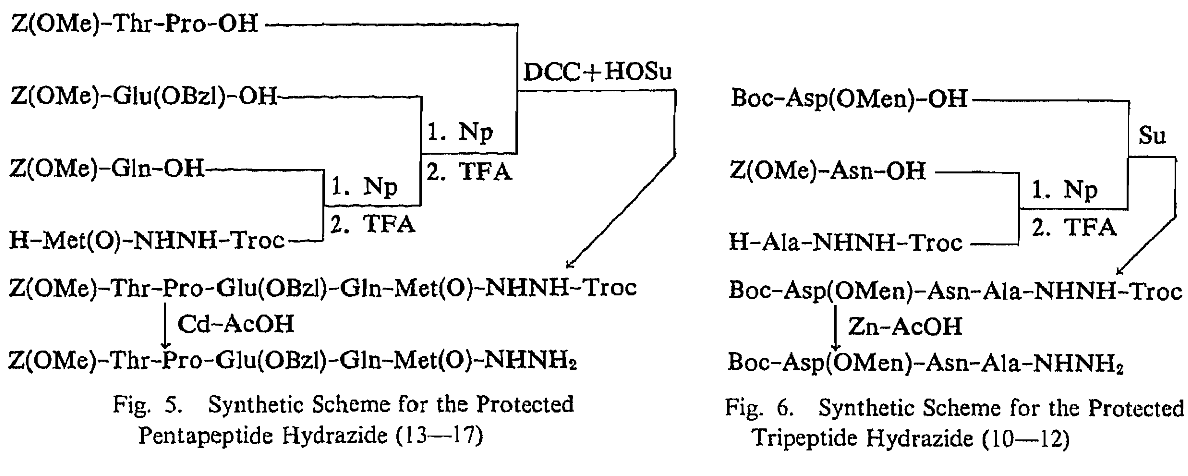

Fig. 5. Synthetic Scheme for the Protected Pentapeptide Hydrazide (13—17)

Fig. 6. Synthetic Scheme for the Protected Tripeptide Hydrazide (10—12)

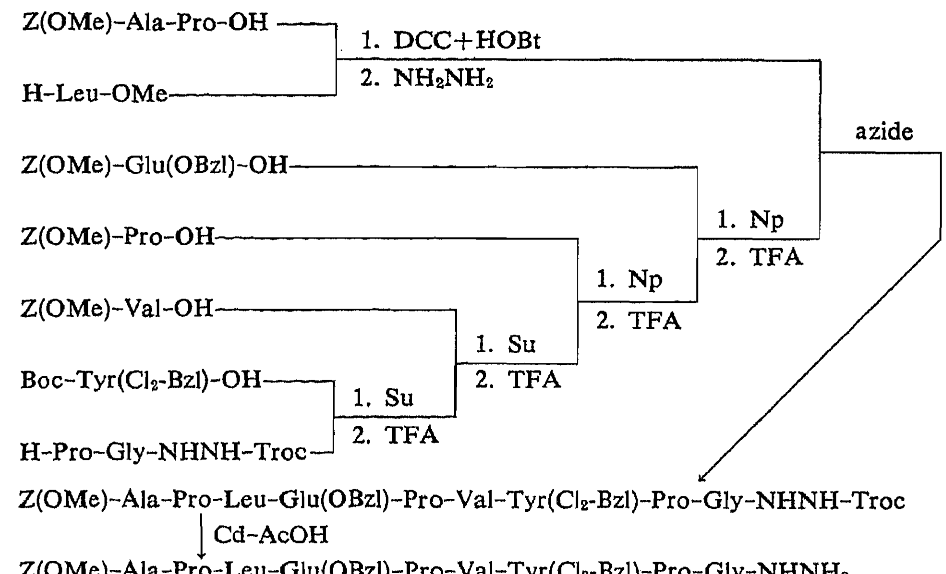

Fig. 7. Synthetic Scheme for the N-Terminal Protected Nonapeptide Hydrazide (1—9)

cedure[27] for Z(OMe)–Thr–Pro–OH.[28] From the resulting protected pentapeptide derivative, the Troc group was removed by treatment with Cd–AcOH.[29] This treatment required a much longer time than Zn–AcOH treatment, but in this case, a more homogeneous product was obtained.

Next, the Asp(OMen)-containing fragment [6], Boc–Asp(OMen)–Asn–Ala–$NHNH_2$ (positions 10—12), was prepared in a stepwise manner by the active ester method, starting with a TFA-treated sample of Z(OMe)–Ala–NHNH–Troc as shown in Fig. 5. The Np and the Su methods were employed to condense Z(OMe)–Asn–OH and Boc–Asp(OMen)–OH, respectively. The Troc group was cleaved from the protected tripeptide derivative by Zn–AcOH treatment within 1 h. During the synthesis, no side products due to base-catalyzed succinimide formation were detected.

The N-terminal nonapeptide hydrazide [7], Z(OMe)–Ala–Pro–Leu–Glu(OBzl)–Pro–Val–Tyr(Cl_2-Bzl)–Pro–Gly–$NHNH_2$ (positions 1—9), was prepared starting with a TFA-treated sample of the known dipeptide derivative, Z(OMe)–Pro–Gly–NHNH–Troc.[30] Up to the hexapeptide stage, the respective amino acid residues, Tyr(Cl_2-Bzl), Val, Pro, and Glu(OBzl), were introduced in a stepwise manner by the Su or the Np method, then the

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic hPP and Its Intermediates

	Protected peptides							Syn. hPP	Residue
	27—36	25—36	22—36	18—36	13—36	10—36	1—36		
Asp	1.03	1.04	2.02	2.12	2.04	3.93	4.25	4.05	4
Thr	0.98	0.93	0.90	1.01	1.87	1.87	2.03	1.92	2
Glu				1.02	2.88	2.95	4.25	3.99	4
Pro	1.01	0.99	1.05	1.09	1.92	2.17	5.37	5.15	5
Gly							1.06	1.11	1
Ala			0.93	3.02	2.93	3.78	5.26	5.14	5
Val							1.03	1.01	1
Met[a]	0.78	0.86	0.62	0.89	1.60	1.57	1.75	1.73	2
Ile[b]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1
Leu	1.06	0.96	1.96	1.99	1.99	2.02	3.06	3.07	3
Tyr	1.92	1.87	1.98	2.78	2.71	2.76	3.83	3.98	4
Arg	2.06	4.08	3.93	4.11	4.04	4.03	4.16	4.20	4
Recovery (%)	88	75	78	70	71	90	95	97	

a) Met+Met(O). *b*) Diagnostic amino acid.

N-terminal tripeptide unit, Z(OMe)–Ala–Pro–Leu–$NHNH_2$ was incorporated by the azide method, as shown in Fig. 7. This tripeptide unit was prepared by DCC–HOBt condensation of Z(OMe)–Ala–Pro–OH[31] with H–Leu–OMe, followed by the usual hydrazine treatment. In the final step, the Troc group was removed from the protected nonapeptide derivative by treatment with Cd–AcOH as was done in the preparation of fragment [7].

Seven peptide fragments thus obtained were then condensed successively by the azide method, according to the route illustrated in Fig. 1. Every reaction was continued until the solution became negative to ninhydrin. The amount of an acyl component was increased from 1 to 3 eq as the chain elongation progressed. Every product was purified by either precipitation from DMF with MeOH or AcOEt or by gel-filtration on Sephadex LH-60. Throughout the synthesis, Ile was used as a diagnostic amino acid in acid hydrolysis (Table I). By comparison of the recovery of Ile with those of newly incorporated amino acids, satisfactory incorporation of each fragment was ascertained.

Protected hPP thus obtained was first treated with phenylthiotrimethylsilane in DMF at room temperature for 60 min to reduce the Met(O) residue.[32] The progress of the reaction was monitored by TLC. The reduced peptide was then treated with 2 M TMSOTf/TFA in the presence of diphenylsulfide (to a concentration of 0.5 M) in an ice-bath for 2 h to remove all protecting groups employed. To ensure the complete removal of the many protecting groups, the amount of TMSOTf in TFA was increased from a concentration of 1 M used in model experiments to 2 M. The deprotected peptide was next treated with dil. ammonia containing NH_4F at pH 8.0 to hydrolyze the trimethylsilyl groups and to reverse any possible N→O shift at the Thr residues. The treated product was purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-Trisacryl using $AcONH_4$ buffer. This procedure was found effective to remove a side product, a succinimide derivative presumably formed during the deprotection. This side product isolated from the tailing portion of the main peak gave a low recovery of Asp in enzymatic digestion. The desired product was finally purified by high-performance liquid chromatography (HPLC) on a Cosmosil $5C_{18}$ column with isocratic elution with MeCN (35—65%, v/v)–0.1% TFA. The product was obtained in 17% yield from protected hPP as a fluffy white powder. Its purity was ascertained by TLC, analytical HPLC, disk isoelectrofocusing, and amino acid analyses after 6 N HCl hydrolysis

TABLE II. Incremental Output (Mean ± S.E.) of Protein during Bile-Pancreatic Juice Diversion with or without Synthetic hPP Infusion

		Protein (mg/h)
Control	(n=8)	20.46 ± 2.66
hPP 1 μg/kg/h	(n=8)	11.47 ± 1.76
hPP 10 μg/kg/h	(n=7)	7.59 ± 2.41

and leucine-aminopeptidase digestion.

When assayed according to Miyasaka *et al.*[33] our synthetic peptide (1 μg/kg/h) inhibited protein secretion from rat pancreas by about 50% as shown in Table II and its potency was judged to be nearly equivalent to that of synthetic rat PP (purchased from Peninsula Co., U.S.A.).

Experimental

General experimental methods employed here are essentially the same as described in Part CXL[34] of this series. The N$^{\alpha}$-protecting group was cleaved by TFA in the presence of anisole as usual. DCC and active ester condensations were performed at room temperature. A mixed anhydride was prepared using isobutyl chloroformate and allowed to react with an amino component in an ice-bath for 5 h. Each hydrazide was converted to the corresponding azide by treatment with isoamyl nitrite and the azide reaction was performed at 4 °C.

Products were purified by one of the following procedures. Procedure A: For purification of a product soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then dried over Na_2SO_4 and concentrated. The residue was recrystallized or precipitated from appropriate solvents. Procedure B: For purification of a peptide less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and recrystallized or precipitated from appropriate solvents. Procedure C: Several fragment condensation products were purified by gel-filtration on Sephadex LH-60 using DMF as an eluant. The fractions corresponding to the front main peak, monitored by ultraviolet (UV) absorption measurement at 275 nm, were combined and the solvent was removed by evaporation. The residue was precipitated from DMF with appropriate solvents.

HPLC was conducted with a Waters 204 compact model. TLC was performed on silica gel (Kiesel-gel 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 : 0.5), Rf_3 $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), Rf_4 n-BuOH–AcOH–pyridine–H_2O: (4 : 1 : 1 : 2), Rf_5 n-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1). Leucine-aminopeptidase was purchased from Sigma Chem. Co. (Lot. No. 62F-8000).

Boc–Tyr(Cl_2-Bzl)–NH_2——Boc–Tyr(Cl_2-Bzl)–OH was converted to the amide by the MA method and the product was purified by precipitation from DMSO–DMF (1 : 1) with AcOEt; yield 4.35 g (87%), mp 216—217 °C, $[\alpha]_D^{19}$ +8.7° (c=1.0, DMSO), Rf_1 0.87. *Anal.* Calcd for $C_{21}H_{24}Cl_2N_2O_4$: C, 57.41; H, 5.51; N, 6.38. Found: C, 57.41; H, 5.61; N, 6.29.

Z(OMe)–Arg(Mts)–Tyr(Cl_2-Bzl)–NH_2——The title compound was prepared by the MA method and purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.97 g (78%), mp 138—140 °C, $[\alpha]_D^{19}$ −13.5° (c=1.0, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{40}H_{46}Cl_2N_6O_8S$: C, 56.81; H, 5.43; N, 9.88. Found: C, 57.07; H, 5.51; N, 9.98.

Boc–Pro–Arg(Mts)–Tyr(Cl_2-Bzl)–NH_2——The MA [prepared from 0.92 g (4.28 mmol) of Boc–Pro–OH] in THF (15 ml) was added to an ice-chilled solution of a TFA-treated sample of the above dipeptide amide (3.0 g, 3.56 mmol) in DMF (30 ml) containing Et_3N (0.50 ml, 3.56 mmol) and the mixture was stirred for 5 h. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 2.53 g (81%), mp 165—167 °C, $[\alpha]_D^{19}$ −22.0° (c=1.0, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{41}H_{53}Cl_2N_7O_8S$: C, 56.29; H, 6.11; N, 11.21. Found: C, 56.02; H, 6.06; N, 10.93.

Z(OMe)–Leu–Thr–$NHNH_2$——Z(OMe)–Leu–Thr–OMe in MeOH was treated with 80% hydrazine hydrate (5 eq) overnight and the product was precipitated from DMF with MeOH; yield 1.45 g (76%), mp 217—219 °C, $[\alpha]_D^{19}$ −2.4° (c=1.0, DMF), Rf_1 0.65. *Anal.* Calcd for $C_{19}H_{30}N_4O_6$: C, 55.60; H, 7.37; N, 13.65. Found: C, 55.70; H, 7.37; N, 13.38.

Z(OMe)–Leu–Thr–Arg(Mts)–$NHNH_2$——The azide [prepared from 1.30 g (3.17 mmol) of Z(OMe)–Leu–Thr–$NHNH_2$] in DMF (15 ml) and Et_3N (0.44 ml, 3.17 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Arg(Mts)–OMe (1.69 g, 3.17 mmol) in DMF (15 ml) containing Et_3N (0.44 ml, 3.17 mmol) and the mixture was stirred overnight. The product was purified by procedure A, then dissolved in MeOH (20 ml), and 80%

hydrazine hydrate (1.0 ml, 5 eq) was added. After standing overnight, the solution was concentrated and the residue was recrystallized from MeOH and ether; yield 1.83 g (77%), mp 166—167 °C, $[\alpha]_D^{19}$ −22.2° (*c*=1.0, MeOH), *Rf*$_1$ 0.67. *Anal.* Calcd for $C_{34}H_{52}N_8O_9S$: C, 54.53; H, 7.00; N, 14.96. Found: C, 54.52; H, 7.12; N, 14.82.

Z(OMe)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$——The azide [prepared from 10.1 g (13.3 mmol) of Z(OMe)–Leu–Thr–Arg(Mts)–NHNH$_2$] in DMF (100 ml) and Et_3N (2.13 ml, 13.3 mmol) were added to an ice-chilled solution of a TFA-treated sample of Boc–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$ (9.75 g, 11.1 mmol) in DMF (100 ml) containing Et_3N (1.78 ml, 11.1 mmol) and the mixture was stirred overnight. The product was purified by procedure B, followed by recrystallization from MeOH and 2-propanol; yield 13.3 g (80%), mp 136—138 °C, $[\alpha]_D^{16}$ −12.5° (*c*=1.0, DMF), *Rf*$_1$ 0.78. *Anal.* Calcd for $C_{70}H_{93}Cl_2N_{13}O_{15}S_2 \cdot 1.5H_2O$: C, 55.36; H, 6.17; N, 11.99. Found: C, 55.53; H, 6.14; N, 11.75.

Z(OMe)–Asn–Met(O)–OMe——The title compound was prepared by the Np method and purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.75 g (70%), mp 184—186 °C, $[\alpha]_D^{16}$ −10.9° (*c*=1.0, DMF), *Rf*$_1$ 0.63. *Anal.* Calcd for $C_{19}H_{27}N_3O_8S$: C, 49.88; H, 5.95; N, 9.18. Found: C, 50.00; H, 5.83; N, 8.98.

Z(OMe)–Ile–Asn–Met(O)–OMe——A mixture of Z(OMe)–Ile–ONp (3.82 g, 9.18 mmol), Et_3N (2.35 ml, 16.83 mmol) and a TFA-treated sample of the above dipeptide ester (3.50 g, 7.65 mmol) in DMF–DMSO (1 : 5, 60 ml) was stirred overnight and the product was purified by procedure B, followed by precipitation from DMF–DMSO (1 : 1) with MeOH; yield 4.13 g (95%), mp 243 °C (dec.), $[\alpha]_D^{16}$ −8.0° (*c*=0.5, DMSO), *Rf*$_1$ 0.59. *Anal.* Calcd for $C_{25}H_{38}N_4O_9S$: C, 52.62; H, 6.71; N, 9.82. Found: C, 52.44; H, 6.79; N, 9.55.

Z(OMe)–Ile–Asn–Met(O)–NHNH$_2$——The above tripeptide ester (4.00 g, 7.01 mmol) in HMPA–DMF (1 : 2, 60 ml) was treated with 80% hydrazine hydrate (2.19 ml, 5 eq) overnight. MeOH was added and the resulting powder was washed with the same solvent; yield 3.90 g (98%), mp 235 °C (dec.), $[\alpha]_D^{16}$ −34.1° (*c*=0.5, DMSO), *Rf*$_1$ 0.27. *Anal.* Calcd for $C_{24}H_{38}N_6O_8S \cdot 1/2H_2O$: C, 49.72; H, 6.78; N, 14.50. Found: C, 49.89; H, 6.77; N, 14.49.

Z(OMe)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$——The azide [prepared from 7.18 g (12.6 mmol) of Z(OMe)–Ile–Asn–Met(O)–NHNH$_2$] in DMF–HMPA (1 : 1, 100 ml) and Et_3N (1.75 ml, 12.6 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$ (12.6 g, 8.42 mmol) in DMF (100 ml) containing Et_3N (1.17 ml, 8.42 mmol) and the mixture was stirred for 48 h. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 12.0 g (76%), mp 186—188 °C, $[\alpha]_D^{16}$ −20.7° (*c*=1.0, DMF), *Rf*$_1$ 0.66. *Anal.* Calcd for $C_{85}H_{119}Cl_2N_{17}O_{20}S_3 \cdot 2H_2O$: C, 53.67; H, 6.52; N, 12.52. Found: C, 53.53; H, 6.23; N, 12.52.

Z(OMe)–Tyr(Cl$_2$-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$ [1] (Positions 27—36)——A mixture of Z(OMe)–Tyr(Cl$_2$-Bzl)–OSu (4.22 g, 7.08 mmol), Et_3N (0.99 ml, 7.08 mmol) and a TFA-treated sample of the above protected nonapeptide amide (11.0 g, 5.89 mmol) in DMF (100 ml) was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 10.43 g (81%), mp 230—232 °C, $[\alpha]_D^{16}$ −20.9° (*c*=1.0, DMF), *Rf*$_1$ 0.62. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. *Anal.* Calcd for $C_{101}H_{132}Cl_4N_{18}O_{22}S_3 \cdot 2H_2O$: C, 54.53; H, 6.16; N, 11.34. Found: C, 54.43; H, 6.01; N, 11.37.

Boc–Asp(OMen)–Leu–NHNH–Troc——The title compound was prepared by the Su method and purified by precipitation from DMF with AcOEt; yield 72%, mp 208 °C (dec.), $[\alpha]_D^{16}$ −74.3° (*c*=0.7, DMF), *Rf*$_2$ 0.89. *Anal.* Calcd for $C_{28}H_{47}Cl_3N_4O_8$: C, 49.89; H, 7.03; N, 8.31. Found: C, 49.65; H, 7.05; N, 8.38.

Z(OMe)–Ala–Asp(OMen)–Leu–NHNH–Troc——A mixture of Z(OMe)–Ala–OSu (2.49 g, 7.12 mmol), Et_3N (1.83 ml, 13.05 mmol) and a TFA-treated sample of Boc–Asp(OMen)–Leu–NHNH–Troc (4.0 g, 5.93 mmol) was stirred overnight and the product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.41 g (92%), mp 98—99 °C, $[\alpha]_D^{16}$ −43.2° (*c*=1.0, DMF), *Rf*$_2$ 0.81. *Anal.* Calcd for $C_{35}H_{52}Cl_3N_5O_{10}$: C, 51.95; H, 6.48; N, 8.31. Found: C, 52.17; H, 6.72; N, 8.34.

Z(OMe)–Ala–Asp(OMen)–Leu–NHNH$_2$ [3] (Position 22—24)——The above protected tripeptide derivative (1.50 g, 1.85 mmol) in AcOH (20 ml) was treated with Zn powder (1.22 g, 10 eq) for 1.5 h. The solution was filtered, the filtrate was concentrated, and the residue was treated with 5% EDTA to form a powder, which was recrystallized from MeOH and 2-propanol; yield 0.80 g (69%), mp 188—189 °C, $[\alpha]_D^{16}$ −63.3° (*c*=1.0, DMF), *Rf*$_1$ 0.74. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.03, Ala 1.05, Leu 1.00 (recovery of Leu, 86%). *Anal.* Calcd for $C_{32}H_{51}N_5O_8$: C, 60.64; H, 8.11; N, 11.05. Found: C, 60.43; H, 8.07; N, 10.98.

Boc–Tyr(Cl$_2$-Bzl)–Ala–OMe——The title compound was prepared by the Su method and purified by procedure A, followed by recrystallization from AcOEt and ether; yield 78%, mp 124—126 °C, $[\alpha]_D^{16}$ −4.5° (*c*=1.0, MeOH), *Rf*$_2$ 0.76. *Anal.* Calcd for $C_{25}H_{30}Cl_2N_2O_6$: C, 57.15; H, 5.75; N, 5.33. Found: C, 57.38; H, 5.73; N, 5.43.

Z(OMe)–Gln–Tyr(Cl$_2$-Bzl)–Ala–OMe——A mixture of Z(OMe)–Gln–ONp (2.95 g, 6.85 mmol), Et_3N (1.74 ml, 12.56 mmol) and a TFA-treated sample of the above dipeptide ester (3.00 g, 5.71 mmol) in DMSO (60 ml) was stirred overnight. The product was purified by procedure B, followed by precipitation from DMSO with MeOH; yield 3.69 g (90%), mp 258—261 °C, $[\alpha]_D^{16}$ −72.7° (*c*=0.5, DMSO), *Rf*$_1$ 0.75. *Anal.* Calcd for $C_{34}H_{38}Cl_2N_4O_9 \cdot 1/2H_2O$: C, 56.20; H, 5.41; N, 7.71. Found: C, 56.15; H, 5.30; N, 7.68.

Z(OMe)–Ala–Gln–Tyr(Cl$_2$-Bzl)–Ala–OMe——A mixture of Z(OMe)–Ala–OSu (2.05 g, 5.85 mmol), Et_3N (1.50 ml, 10.73 mmol) and a TFA-treated sample of the above tripeptide ester (3.50 g, 4.88 mmol) was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF–DMSO (1 : 1)

with MeOH; yield 3.49 g (91%), mp 268—271 °C, $[\alpha]_D^{16}$ −6.0° (c=0.5, DMSO), Rf_1 0.72. *Anal.* Calcd for $C_{37}H_{43}Cl_2N_5O_{10}\cdot 1.5H_2O$: C, 54.48; H, 5.50; N, 8.58. Found: C, 54.75; H, 5.53; N, 8.36.

Z(OMe)–Ala–Gln–Tyr(Cl_2-Bzl)–Ala–$NHNH_2$ [4] (Position 18—21)——The above protected tetrapeptide ester (2.50 g, 3.17 mmol) in DMF–HMPA (1 : 1, 50 ml) was treated with 80% hydrazine hydrate (0.99 ml, 5 eq) overnight. H_2O was added and the resulting powder was washed thoroughly with MeOH; yield 2.18 g (87%), mp 269—271 °C, $[\alpha]_D^{16}$ −4.1° (c=0.5, DMSO), Rf_1 0.60. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.00, Ala 2.03, Tyr 0.95 (recovery of Glu, 77%). *Anal.* Calcd for $C_{36}H_{43}Cl_2N_7O_9$: C, 54.82; H, 5.50; N, 12.43. Found: C, 54.75; H, 5.54; N, 12.27.

Z(OMe)–Gln–Met(O)–NHNH–Troc——The title compound was prepared by the Np method and purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.60 g (70%), mp 201—202 °C, $[\alpha]_D^{16}$ −17.5° (c=1.0, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{22}H_{30}Cl_3N_5O_9S$: C, 40.84; H, 4.67; N, 10.83. Found: C, 40.67; H, 4.50; N, 10.75.

Z(OMe)–Glu(OBzl)–Gln–Met(O)–NHNH–Troc——Z(OMe)–Glu(OBzl)–ONp (4.85 g, 9.27 mmol) and NMM (1.02 ml, 9.27 mmol) were added to a solution of a TFA-treated sample of the above dipeptide derivative (5.0 g, 7.73 mmol) in DMF (80 ml) containing Et_3N (1.08 ml, 7.73 mmol) and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.40 g (81%), mp 180—182 °C, $[\alpha]_D^{16}$ −12.6° (c=1.0, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{34}H_{43}Cl_3N_6O_{12}S$: C, 47.15; H, 5.00; N, 9.70. Found: C, 47.24; H, 4.74; N, 9.71.

Z(OMe)–Thr–Pro–Glu(OBzl)–Gln–Met(O)–NHNH–Troc——Z(OMe)–Thr–Pro–OSu [prepared from 1.32 g (3.46 mmol) of Z(OMe)–Thr–Pro–OH] and NMM (0.38 ml, 3.46 mmol) were added to a solution of a TFA-treated sample of the above tripeptide derivative (2.50 g, 2.89 mmol) in DMF (30 ml) containing Et_3N (0.40 ml, 2.89 mmol) and the mixture was stirred overnight. The product was purified by procedure A, followed by column chromatography on silica gel (6×7.3 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was triturated with AcOEt and ether; yield 2.10 g (68%), mp 115—117 °C, $[\alpha]_D^{16}$ −53.8° (c=1.0, MeOH), Rf_1 0.64. *Anal.* Calcd for $C_{43}H_{57}Cl_3N_8O_{15}S\cdot 1/2H_2O$: C, 48.11; H, 5.35; N, 10.44. Found: C, 47.97; H, 5.43; N, 10.20.

Z(OMe)–Thr–Pro–Glu(OBzl)–Gln–Met(O)–$NHNH_2$ [5] (Positions 13—17)——The above protected pentapeptide derivative (1.10 g, 1.03 mmol) in DMF–AcOH (20 ml–1 ml) was treated with Cd powder (2.32 g, 20.6 mmol) for 72 h and the solution was filtered. The filtrate was concentrated and the residue was dissolved in n-BuOH. The organic phase was washed with 5% EDTA and H_2O and concentrated. Treatment of the residue with ether afforded a powder; yield 750 mg (82%), mp 152—154 °C, $[\alpha]_D^{16}$ −28.0° (c=1.0, DMF), Rf_1 0.40. Amino acid ratios in a 6 N HCl hydrolysate: Thr 1.00, Glu 2.08, Pro 0.95, Met+Met(O) 0.71 (recovery of Thr, 76%). *Anal.* Calcd for $C_{40}H_{56}N_8O_{13}S\cdot 1/2H_2O$: C, 53.50; H, 6.40; N, 12.48. Found: C, 53.49; H, 6.32; N, 12.22.

Z(OMe)–Asn–Ala–NHNH–Troc——The title compound was prepared by the Np method and purified by procedure B, followed by precipitation from DMF with MeOH; yield 66%, mp 195—197 °C, $[\alpha]_D^{19}$ −14.8° (c=1.0, DMF), Rf_1 0.63. *Anal.* Calcd for $C_{19}H_{24}Cl_3N_5O_8$: C, 40.99; H, 4.34; N, 12.58. Found: C, 41.09; H, 4.42; N, 12.58.

Boc–Asp(OMen)–Asn–Ala–NHNH–Troc——Boc–Asp(OMen)–OSu (5.05 g, 10.8 mmol) and NMM (1.18 ml, 10.8 mmol) were added to a solution of a TFA-treated sample of the above dipeptide derivative (5.0 g, 8.98 mmol) in DMF (80 ml) containing Et_3N (1.25 ml, 8.98 mmol) and the mixture was stirred overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 4.84 g (72%), mp 123—125 °C, $[\alpha]_D^{19}$ −33.8° (c=1.0, MeOH), Rf_1 0.79. *Anal.* Calcd for $C_{29}H_{47}Cl_3N_6O_{10}$: C, 46.69; H, 6.35; N, 11.26. Found: C, 46.86; H, 6.42; N, 11.13.

Boc–Asp(OMen)–Asn–Ala–$NHNH_2$ [6] (Positions 10—12)——The above tripeptide derivative (2.50 g, 3.35 mmol) in AcOH (30 ml) was treated with Zn powder (2.21 g, 10 eq) for 1 h. The solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% EDTA and H_2O, dried over Na_2SO_4 and concentrated. The residue was recrystallized from MeOH and ether; yield 1.66 g (87%), mp 170—172 °C, $[\alpha]_D^{19}$ −47.6° (c=1.0, MeOH), Rf_1 0.63. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.04, Ala 1.00 (recovery of Ala, 93%). *Anal.* Calcd for $C_{26}H_{46}N_6O_8\cdot 2H_2O$: C, 51.47; H, 7.64; N, 13.85. Found: C, 51.75; H, 7.63; N, 14.05.

Boc–Tyr(Cl_2-Bzl)–Pro–Gly–NHNH–Troc——A mixture of Boc–Tyr(Cl_2-Bzl)–OSu (8.38 g, 15.6 mmol), Et_3N (3.98 ml, 28.6 mmol), and a TFA-treated sample of Z(OMe)–Pro–Gly–NHNH–Troc (6.83 g, 13.0 mmol) in DMF (100 ml) was stirred overnight and the product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 8.19 g (80%), mp 168—170 °C, $[\alpha]_D^{16}$ −16.5° (c=1.0, MeOH), Rf_1 0.68. *Anal.* Calcd for $C_{31}H_{36}Cl_5N_5O_8$: C, 47.50; H, 4.63; N, 8.93. Found: C, 47.71; H, 4.73; N, 8.64.

Z(OMe)–Val–Tyr(Cl_2-Bzl)–Pro–Gly–NHNH–Troc——A mixture of Z(OMe)–Val–OSu (2.03 g, 5.36 mmol), Et_3N (1.37 ml, 9.82 mmol) and a TFA-treated sample of the above tripeptide derivative (3.50 g, 4.46 mmol) in DMF (50 ml) was stirred overnight. The product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 2.84 g (67%), mp 122—124 °C, $[\alpha]_D^{16}$ −35.8° (c=1.0, MeOH), Rf_1 0.70. *Anal.* Calcd for $C_{40}H_{45}Cl_5N_6O_{10}\cdot 1/2H_2O$: C, 50.24; H, 4.85; N, 8.79. Found: C, 49.96; H, 4.70; N, 9.10.

Z(OMe)–Pro–Val–Tyr(Cl_2-Bzl)–Pro–Gly–NHNH–Troc——A mixture of Z(OMe)–Pro–ONp [prepared from 1.20 g (3.17 mmol) of the CHA salt], Et_3N (0.81 ml, 5.81 mmol) and a TFA-treated sample of the above tetrapeptide

derivative (2.50 g, 2.64 mmol) in DMF (30 ml) was stirred overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 1.95 g (71%), mp 119—121 °C, $[\alpha]_D^{16}$ $-53.0°$ (c=1.0, MeOH), Rf_1 0.79. *Anal.* Calcd for $C_{45}H_{52}Cl_5N_7O_{11}$: C, 51.76; H, 5.02; N, 9.39. Found: C, 51.53; H, 5.07; N, 9.35.

Z(OMe)–Glu(OBzl)–Pro–Val–Tyr(Cl$_2$-Bzl)–Pro–Gly–NHNH–Troc——Z(OMe)–Glu(OBzl)–ONp (1.65 g, 3.16 mmol) and NMM (0.35 ml, 3.16 mmol) were added to a solution of a TFA-treated sample of the above pentapeptide derivative (2.75 g, 2.63 mmol) in DMF (50 ml) containing Et_3N (0.37 ml, 2.63 mmol) and the mixture was stirred overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 2.98 g (90%), mp 110—111 °C, $[\alpha]_D^{16}$ $-69.4°$ (c=1.0, MeOH), Rf_1 0.87. *Anal.* Calcd for $C_{57}H_{65}Cl_5N_8O_{14}$: C, 54.19; H, 5.19; N, 8.87. Found: C, 53.89; H, 5.15; N, 8.97.

Z(OMe)–Ala–Pro–Leu–NHNH$_2$——DCC (8.37 g, 40.6 mmol) was added to a mixture of Z(OMe)–Ala–Pro–OH (11.84 g, 33.8 mmol), HOBt (5.5 g, 40.6 mmol) and H–Leu–OMe [prepared from 6.14 g (33.8 mmol) of the HCl salt] in DMF (100 ml). The solution was stirred for 18 h and filtered. The solvent was removed by evaporation and the residue was purified by procedure A to give an oily product; yield 14.37 g (89%). This ester was dissolved in MeOH (100 ml) and treated with 80% hydrazine hydrate (7.54 ml, 5 eq) overnight. The solvent was evaporated off and the residue was dissolved in AcOEt. The organic phase was washed with H_2O, dried over Na_2SO_4 and concentrated. Trituration of the residue with n-hexane afforded a powder; yield 13.51 g (94%), mp 70—72 °C, $[\alpha]_D^{20}$ $-87.6°$ (c=0.7, MeOH), Rf_1 0.66. Amino acid ratios in a 6 N HCl hydrolysate: Pro 0.98, Ala 0.99, Leu 1.00 (recovery of Leu, 82%). *Anal.* Calcd for $C_{23}H_{35}N_5O_6 \cdot 1/2H_2O$: C, 56.77; H, 7.46; N, 14.39. Found: C, 57.42; H, 7.75; N, 14.07.

Z(OMe)–Ala–Pro–Leu–Glu(OBzl)–Pro–Val–Tyr(Cl$_2$-Bzl)–Pro–Gly–NHNH–Troc——The azide, prepared from Z(OMe)–Ala–Pro–Leu–NHNH$_2$ (1.13 g, 2.37 mmol), in DMF (10 ml) and NMM (0.26 ml, 2.37 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected hexapeptide derivative (2.50 g, 1.98 mmol) in DMF (30 ml) containing Et_3N (0.28 ml, 1.98 mmol) and the mixture was stirred at 4 °C overnight. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 2.73 g (89%), mp 133—134 °C, $[\alpha]_D^{16}$ $-73.9°$ (c=1.0, MeOH), Rf_1 0.75, Rf_2 0.54. *Anal.* Calcd for $C_{71}H_{88}Cl_5N_{11}O_{17}$: C, 55.20; H, 5.74; N, 9.97. Found: C, 54.94; H, 5.76; N, 9.91.

Z(OMe)–Ala–Pro–Leu–Glu(OBzl)–Pro–Val–Tyr(Cl$_2$-Bzl)–Pro–Gly–NHNH$_2$ [7] (Positions 1—9)——The above Troc derivative (1.25 g, 0.81 mmol) in DMF–AcOH (10 ml–1 ml) was treated with Cd powder (1.8 g, 20 eq) at room temperature for 3 h. The solution was filtered, the filtrate was concentrated *in vacuo* and the residue was treated with 5% EDTA. The resulting powder was washed with 5% EDTA, 5% $NaHCO_3$ and H_2O and recrystallized from MeOH and ether; yield 0.90 g (81%), mp 141—143 °C, $[\alpha]_D^{16}$ $-79.7°$ (c=1.0, MeOH), Rf_1 0.83, Rf_2 0.38. Amino acid ratios in a 6 N HCl hydrolysate: Glu 0.95, Pro 3.10, Gly 1.00, Ala 0.95, Val,0.93, Leu 0.98, Tyr 0.92 (recovery of Gly, 91%). *Anal.* Calcd for $C_{68}H_{87}Cl_2N_{11}O_{15} \cdot 3H_2O$: C, 57.37; H, 6.59; N, 10.82. Found: C, 57.56; H, 6.26; N, 10.75.

Z(OMe)–Arg(Mts)–Arg(Mts)–Tyr(Cl$_2$-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$, Z(OMe)–(hPP 25—36)–NH$_2$——The azide, prepared from fragment [2] (2.40 g, 2.74 mmol), in DMF (30 ml) and Et_3N (0.38 ml, 2.74 mmol) were added to an ice-chilled solution of a TFA-treated sample of fragment [1] (4.0 g, 1.83 mmol) in DMF (40 ml) containing Et_3N (0.26 ml, 1.83 mmol) and the mixture was stirred overnight. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.68 g (89%), mp 184—185 °C, $[\alpha]_D^{16}$ $-16.3°$ (c=1.0, DMF), Rf_1 0.64. *Anal.* Calcd for $C_{131}H_{176}Cl_4N_{26}O_{28}S_5 \cdot 3H_2O$: C, 53.89; H, 6.28; N, 12.48. Found: C, 53.88; H, 6.26; N, 12.65.

Z(OMe)–Ala–Asp(OMen)–Leu–Arg(Mts)–Arg(Mts)–Tyr(Cl$_2$-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$, Z(OMe)–(hPP 22—36)–NH$_2$——The azide, prepared from fragment [3] (1.49 g, 2.35 mmol), in DMF (20 ml) and Et_3N (0.33 ml, 2.35 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(hPP 25—36)–NH$_2$ (4.48 g, 1.56 mmol) in DMF (30 ml) containing Et_3N (0.22 ml, 1.56 mmol) and the mixture was stirred for 48 h. The product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 3.55 g (69%), mp 149—151 °C, $[\alpha]_D^{16}$ $-13.2°$ (c=1.0, DMF), Rf_1 0.71. *Anal.* Calcd for $C_{154}H_{215}Cl_4N_{29}O_{33}S_5 \cdot 5H_2O$: C, 54.51; H, 6.68; N, 11.97. Found: C, 54.54; H, 6.64; N, 11.75.

Z(OMe)–Ala–Gln–Tyr(Cl$_2$-Bzl)–Ala–Ala–Asp(OMen)–Leu–Arg(Mts)–Arg(Mts)–Tyr(Cl$_2$-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$, Z(OMe)–(hPP 18—36)–NH$_2$——The azide, prepared from fragment [4] (1.17 g, 1.49 mmol), in DMF–DMSO (1 : 1, 20 ml) and Et_3N (0.21 ml, 1.49 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(hPP 22—36)–NH$_2$ (3.28 g, 0.99 mmol) in DMF (30 ml) containing Et_3N (0.14 ml, 0.99 mmol) and the mixture was stirred overnight. The product was purified by procedure B, followed by gel-filtration on Sephadex LH-60 (3 × 147 cm) using DMF as an eluant. The product was finally precipitated from DMF with MeOH; yield 3.27 g (85%), mp 154—155 °C, $[\alpha]_D^{16}$ $-14.1°$ (c=1.0, DMF), Rf_1 0.6. *Anal.* Calcd for $C_{181}H_{246}Cl_6N_{34}O_{39}S_5 \cdot 5H_2O$: C, 54.55; H, 6.47; N, 11.95. Found: C, 54.35; H, 6.18; N, 11.78.

Z(OMe)–Thr–Pro–Glu(OBzl)–Gln–Met(O)–Ala–Gln–Tyr(Cl$_2$-Bzl)–Ala–Ala–Asp(OMen)–Leu–Arg(Mts)–Arg(Mts)–Tyr(Cl$_2$-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl$_2$-Bzl)–NH$_2$, Z(OMe)–(hPP 13—36)–NH$_2$——The azide, prepared from fragment [5] (1.49 g, 1.68 mol), in DMF (20 ml) and NMM (0.18 ml, 1.68 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(hPP 18—36)–NH$_2$ (3.27 g, 0.84 mmol) in DMF (30 ml) containing Et_3N (0.12 ml, 0.84 mmol) and the mixture was stirred overnight. The product was purified by procedure B, followed by gel-filtration on Sephadex LH-60 (3 × 147 cm) using DMF as an eluant. The

product was finally precipitated from DMF with MeOH; yield 3.36 g (87%), mp 157—159 °C, $[\alpha]_D^{16}$ −8.1° (c=1.0, DMF), Rf_1 0.61. *Anal.* Calcd for $C_{212}H_{290}Cl_6N_{40}O_{49}S_6 \cdot 6H_2O$: C, 54.22; H, 6.48; N, 11.93. Found: C, 54.00; H, 6.33; N, 11.82.

Boc–Asp(OMen)–Asn–Ala–Thr–Pro–Glu(OBzl)–Gln–Met(O)–Ala–Gln–Tyr(Cl_2-Bzl)–Ala–Ala–Asp(OMen)–Leu–Arg(Mts)–Arg(Mts)–Tyr(Cl_2-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl_2-Bzl)–NH_2, Boc–(hPP 10—36)–NH_2——The azide, prepared from fragment [6] (1.04 g, 1.83 mmol), in DMF (10 ml) and NMM (0.26 ml, 1.83 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(hPP 13—36)–NH_2 (3.36 g, 0.73 mmol) in DMF (30 ml) containing Et_3N (0.10 ml, 0.73 mmol) and the mixture was stirred for 48 h. The product was purified by gel-filtration on Sephadex LH-60 (3 × 137 cm) using DMF as an eluant and finally precipitated from DMF with MeOH; yield 2.89 g (80%), mp 162—163 °C, $[\alpha]_D^{16}$ −6.8° (c=0.5, DMF), Rf_1 0.57. *Anal.* Calcd for $C_{229}H_{324}N_{44}O_{54}S_6 \cdot 10H_2O$: C, 53.48; H, 6.74; N, 11.99. Found: C, 53.17; H, 6.64; N, 12.19.

Z(OMe)–Ala–Pro–Leu–Glu(OBzl)–Pro–Val–Tyr(Cl_2-Bzl)–Pro–Gly–Asp(OMen)–Asn–Ala–Thr–Pro–Glu(OBzl)–Gln–Met(O)–Ala–Gln–Tyr(Cl_2-Bzl)–Ala–Ala–Asp(OMen)–Leu–Arg(Mts)–Arg(Mts)–Tyr(Cl_2-Bzl)–Ile–Asn–Met(O)–Leu–Thr–Arg(Mts)–Pro–Arg(Mts)–Tyr(Cl_2-Bzl)–NH_2, Z(OMe)–(1—36)–NH_2——The azide, prepared from fragment [7] (2.39 g, 1.75 mmol), in DMF (20 ml) and NMM (0.19 ml, 1.75 mol) were added to an ice-chilled solution of a TFA-treated sample of Boc–(hPP 10—36)–NH_2 (2.89 g, 0.58 mmol) in DMF (30 ml) containing Et_3N (80 µl, 0.58 mmol) and the mixture was stirred for 48 h. The product was purified by gel-filtration on Sephadex LH-60 (3 × 144 cm) using DMF as an eluant and finally precipitated from DMF with AcOEt; yield 2.86 g (79%), mp 164—168 °C, $[\alpha]_D^{16}$ −40.0° (c=0.5, DMF), Rf_1 0.69. *Anal.* Calcd for $C_{292}H_{399}Cl_8N_{53}O_{67}S_6 \cdot 12H_2O$: C, 54.67; H, 6.65; N, 11.57. Found: C, 54.57; H, 6.55; N, 11.84.

H–Ala–Pro–Leu–Glu–Pro–Val–Tyr–Pro–Gly–Asp–Asn–Ala–Thr–Pro–Glu–Gln–Met–Ala–Gln–Tyr–Ala–Ala–Asp–Leu–Arg–Arg–Tyr–Ile–Asn–Met–Leu–Thr–Arg–Pro–Arg–Tyr–NH_2, Synthetic hPP——Protected hPP obtained above (200 mg, 32.3 µmol) in DMF (2 ml) was treated with phenylthiotrimethylsilane (1.18 ml, 200 eq) at room temperature for 60 min under an N_2 atmosphere, while the starting material disappeared and a new spot, Rf_1 0.78, was detected on TLC. The solution was concentrated and the residue was treated with AcOEt to form a powder; yield 189 mg (95%).

The reduced peptide (50 mg, 8.1 µmol) was treated with 2 M TMSOTf/TFA (4.6 ml) in the presence of diphenylsulfide (to a concentration of 0.5 M) and *m*-cresol (51 µl) in an ice-bath for 120 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in MeOH–H_2O (1 : 1, 4 ml) containing 2-mercaptoethanol (200 µl). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH and 1 M NH_4F (200 µl) was added. After 30 min, the pH was adjusted to 6.0 with 1 N AcOH and the solution was applied to a column of Sephadex G-25 (2.2 × 136 cm), which was eluted with 1 N AcOH. The fractions (6 ml each) corresponding to the front main peak (tube Nos. 37—49, monitored by UV measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a powder; yield 33.3 mg (98%).

The crude product thus obtained was dissolved in H_2O (3 ml) and the solution was applied to a column of CM-Trisacryl (2.0 × 3.5 cm), which was eluted with pH 6.0, 0.2 M $AcONH_4$ buffer (300 ml) through a mixing flask containing pH 6.0, 0.01 M $AcONH_4$ buffer (200 ml) (Fig. 8). The desired fractions (5 ml each, tube Nos. 31—40, monitored by UV measurement at 275 nm) were combined and the solvent and the salt were removed by repeated lyophilization to give a white powder; yield 10.8 mg (32%). The product obtained from the tailing portion of the main peak (5.4 mg) gave a low recovery of Asp in the LAP digest: Asp(2 residues)/Ile(diagnostic amino acid) = 1.41/1.00.

The CM-purified product was next purified by HPLC on a Cosmosil 5C_{18} column (10 × 250 mm) with isocratic elution with MeCN–0.1% TFA (v/v 35 : 65) at a flow rate of 1.8 ml/min (Fig. 9a). The eluate corresponding to the main peak (retention time 25.5 min, monitored by UV measurement at 275 nm) was collected and the solvent was removed by lyophilization. The resulting powder was dissolved in 1 N AcOH (0.5 ml) and subjected to gel-filtration

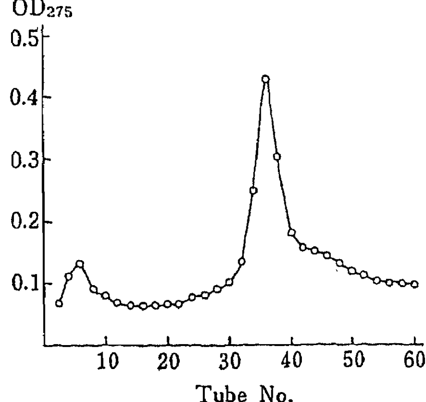

Fig. 8. CM-Trisacryl Purification of Synthetic hPP

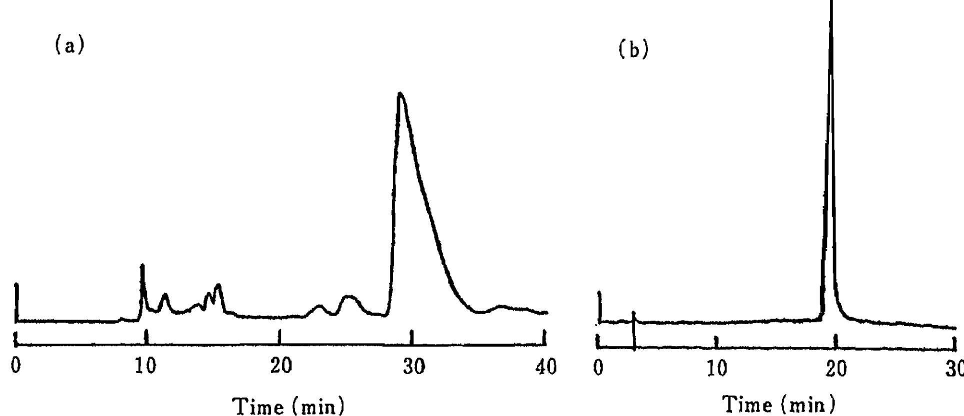

Fig. 9. HPLC of Synthetic hPP (OD at 275 nm)

(a) CM-purified sample on a cosmosil column. (b) Cosmosil-purified sample on a Vydac column.

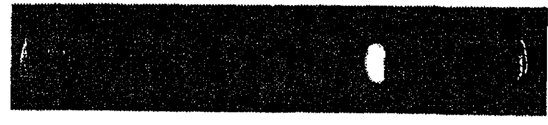

Fig. 10. Disk Isoelectrofocusing of Synthetic hPP

on Sephadex G-25 (1.5 x 38 cm) using 1 N AcOH as an eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 5.1 mg (16.5% from the reduced form of protected hPP). $[\alpha]_D^{19}$ $-48.9°$ ($c=0.1$, H_2O); Rf_4 0.40, Rf_5 0.40; retention time 19.0 min in HPLC on an analytical Vydac $5C_{18}$ column (4.6 x 250 mm) on gradient elution with MeCN (25—45%, 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min (Fig. 9b); a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 x 8.0 cm) containing Pharmalyte (pH 3—10), mobility 5.5 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (Fig. 10). Amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 1.88 (2), Thr 1.66 (2), Glu 1.44 (2), Pro 4.11 (5), Gly 0.98 (1), Ala 4.64 (5), Val 0.98 (1), Met 1.99 (2), Ile 1.00 (1), Leu 2.87 (3), Tyr 3.62 (4), Arg 3.95 (4), Gln and Asn were not determined (recovery of Ile 94%. Because of the presence of 5 Pro residues, complete digestion was not achieved).

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Cl_2-Bzl = 2,6-dichlorobenzyl, Men = *l*-mentyl, Mts = mesitylenesulfonyl, DCC = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, Np = *p*-nitrophenyl, Su = hydroxysuccinimidyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TFMSA = trifluoromethanesulfonic acid, DMF = dimethylformamide, CM = carboxymethyl, HMPA = hexamethylphosphoramide, DMSO = dimethyl sulfoxide, NMM = *N*-methylmorpholine, CHA = cyclohexylamine, Boc = *tert*-butoxycarbonyl, Troc = 2,2,2-trichloroethyloxycarbonyl, EDTA = ethylenediaminetetraacetic acid disodium salt.
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Homoisoflavonoids and Related Compounds. IV.[1] Absolute Configurations of Homoisoflavonoids from *Caesalpinia sappan* L.

MICHIO NAMIKOSHI* and TAMOTSU SAITOH

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

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The absolute configuration of the homoisoflavonoidal components, 4-*O*-methylsappanol, 4-*O*-methylepisappanol, sappanone B, 3-deoxysappanone B and 3′-deoxysappanone B, from *Caesalpinia sappan* L. were determined on the basis of chemical transformations and the optical properties.

Keywords——*Caesalpinia sappan*; Leguminosae; Sappan Lignum; heartwood; homoisoflavonoid; absolute configuration

In the course of our studies on homoisoflavonoids and related compounds, we have investigated the chemical constituents of *Caesalpinia sappan* L.[2-4] and *C. japonica* SIEB. *et* ZUCC.[1] (Leguminosae). *C. sappan* and *C. japonica* contain various types of phenolic compounds, such as chalcones,[1,2,5] flavonoids,[2,6] homoisoflavonoids,[1-4] brazilins,[1,3,7] dibenzoxocin derivatives[1,8] and so on.[9]

Recently,[3] we have reported the isolation and absolute structures of seven components,

1: $R_1=R_2=H$
2: $R_1=Me$, $R_2=H$
3: $R_1=R_2=Me$

4: $R_1=OH$, $R_2=R_3=H$
6: $R_1=OH$, $R_2=H$, $R_3=Me$
8: $R_1=OMe$, $R_2=Me$, $R_3=H$
10: $R_1=OMe$, $R_2=R_3=Me$
12: $R_1=R_2=R_3=H$
14: $R_1=R_3=H$, $R_2=Me$
16: $R_1=R_2=H$, $R_3=Me$
17: $R_1=OMe$, $R_2=R_3=H$

5: $R_1=OH$, $R_2=R_3=H$
7: $R_1=OH$, $R_2=H$, $R_3=Me$
9: $R_1=OMe$, $R_2=Me$, $R_3=H$
11: $R_1=OMe$, $R_2=R_3=Me$
13: $R_1=R_2=R_3=H$
15: $R_1=R_3=H$, $R_2=Me$
18: $R_1=OMe$, $R_2=R_3=H$

19: $R_1=R_3=OH$, $R_2=H$
20: $R_1=OMe$, $R_2=Me$, $R_3=OH$
21: $R_1=OMe$, $R_2=Me$, $R_3=OMs$
22: $R_1=OH$, $R_2=R_3=H$
24: $R_1=OMe$, $R_2=Me$, $R_3=H$
26: $R_1=R_2=H$, $R_3=OH$
27: $R_1=H$, $R_2=Me$, $R_3=OH$

23: R=H
25: R=Me

28

Chart 1

brazilin (**1**), 3′-*O*-methylbrazilin (**2**), sappanol (**4**), episappanol (**5**), 3′-deoxysappanol (**12**), 3′-*O*-methylsappanol (**17**), and 3′-*O*-methylepisappanol (**18**), from Sappan Lignum, the dried heartwood of *C. sappan*. In this paper, we wish to report the absolute structures of 4-*O*-methylsappanol (**6**), 4-*O*-methylepisappanol (**7**), sappanone B (**19**), 3-deoxysappanone B (**22** and **23**) and 3′-deoxysappanone B (**27**) obtained from the same source.

4-*O*-Methylsappanol (6) and 4-*O*-Methylepisappanol (7)

4-*O*-Methylsappanol (**6**)[1,4] and 4-*O*-methylepisappanol (**7**)[1,4] were readily transformed into brazilin (**1**)[1,2] upon heating in MeOH in the presence of an acid. On methylation with diazomethane (CH_2N_2), 4-*O*-methylsappanol (**6**) and 4-*O*-methylepisappanol (**7**) afforded the trimethyl derivatives (**10** and **11**), respectively, which both gave the trimethyl ether of brazilin (**3**)[2] on similar acid treatment. Therefore, the absolute configuration at the C-3 position was determined to be (*R*) for both **6** and **7**.

The proton nuclear magnetic resonance (^{1}H-NMR) signals (observed at 100 MHz) due to the methylene protons at C-9 were characteristic for each of the 3,4-*cis*- and 3,4-*trans*-di-*O*-substituted homoisoflavans, as summarized in Table I. The signals of 3,4-*cis* compounds, such as **4**, **8**, **12**, **14**, **16** and **17** were observed as singlets, while the signals of 3,4-*trans* compounds, **5**, **9**, **13**, **15** and **18**, appeared as geminal-coupled AA′-type doublets. From this observation, 4-*O*-methylsappanol (**6**) could be deduced to have 3,4-*cis* configuration, because the signal due to the methylene protons at C-9 was observed as a singlet at δ 2.56. Moreover, the trimethyl derivative (**10**) of **6** showed the corresponding signal at δ 2.71 as a singlet. On the other hand, the C-9 methylene proton signals of 4-*O*-methylepisappanol (**7**) and its trimethyl derivative (**11**) were observed as pairs of doublets (J=14.0 Hz) at δ 2.63 and 2.89, and at δ 2.77 and 3.04, respectively. Consequently, 4-*O*-methylepisappanol (**7**) has the 3,4-*trans* configuration.

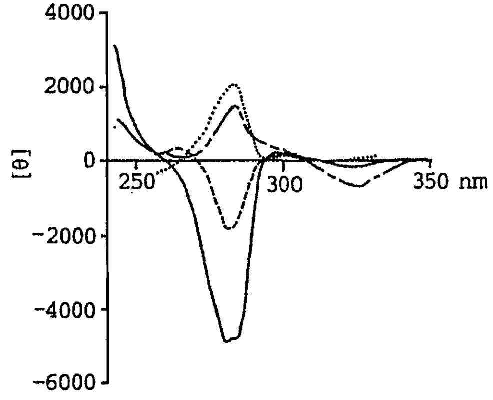
To confirm these conclusions, the circular dichroism (CD) spectra of **10** and **11** were compared with those of **8** and **9**[3] in the region of 250—300 nm. A negative Cotton effect was observed in the CD curves of **8** and **10**, while a positive Cotton effect was seen in those of **9** and **11** (Fig. 1). Therefore, the absolute configuration at C-4 of 4-*O*-methylsappanol (**6**) and 4-*O*-methylepisappanol (**7**) should be (*S*) and (*R*), respectively.

Accordingly, the structures of 4-*O*-methylsappanol (**6**) and 4-*O*-methylepisappanol (**7**) are (3*R*,4*S*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)-4-methoxychroman and (3*R*,4*R*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)-4-methoxychroman, respectively, as shown in Chart 1. The characteristics of the ^{1}H-NMR signals due to the methylene protons at C-9 of 3,4-*cis* and

TABLE I. ^{1}H-NMR Data for the Methylene Protons at C-9 of the 3,4-*cis* Compounds (**4**, **6**, **8**, **10**, **12**, **14** and **15**) and 3,4-*trans* Compounds (**5**, **7**, **9**, **11**, **13** and **16**) (100 MHz)[a]

3,4-*cis* Compounds (2H, s)		3,4-*trans* Compounds (each 1H, d)	
4 (CD_3OD)	2.61	**5** (CD_3OD)	2.67, 2.84 J=14.0
6 (ACTN)[b]	2.56	**7** (ACTN)	2.63, 2.89 J=14.0
8 ($CDCl_3$)	2.76	**9** ($CDCl_3$)	2.72, 3.00 J=14.0
10 ($CDCl_3$)	2.71	**11** ($CDCl_3$)	2.77, 3.04 J=14.0
12 (ACTN)	2.68	**13** (CD_3OD)	2.71, 2.93 J=14.0
14 ($CDCl_3$)	2.78	**15** ($CDCl_3$)	2.75, 3.03 J=14.0
16 (ACTN)	2.60		
17 (CD_3OD)	2.62	**18** (CD_3OD)	2.72, 2.91 J=14.0

a) Chemical shifts are given in δ (ppm) relative to tetramethylsilane. Coupling constants are given in Hz. *b*) ACTN = acetone-d_6.



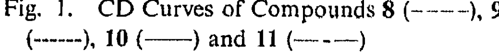


Fig. 1. CD Curves of Compounds **8** (----), **9** (------), **10** (——) and **11** (—-—)

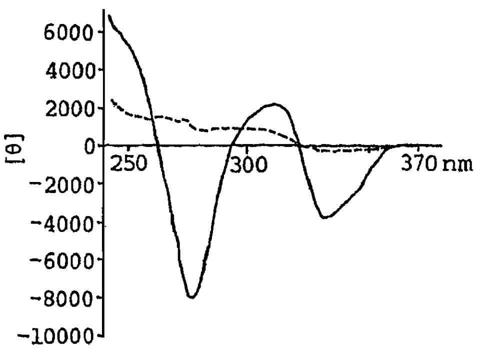


Fig. 2. CD Curves of Compound **25** (——) and the Mixture of Compounds **24** and **25** (----) Derived from the Natural Product (3-Deoxysappanone B)

3,4-*trans* compounds thus appear to be useful for evaluating the stereochemistries of 3,4-di-*O*-substituted homoisoflavans.

It should be noted that the use of a prolonged reaction time on methylation with CH_2N_2 caused epimerization at the C-4 position and the use of EtOH as a solvent resulted in the formation of 4-*O*-ethylated compounds. When the methylation was performed with dimethyl sulfate (Me_2SO_4) in refluxing acetone (1 h), a significant amount of **3** was formed.

Sappanone B (19)

The absolute configuration at the C-3 position of sappanone B (**19**)[1,4] was determined to be (*R*) from the following evidence. Sappanone B (**19**) was transformed into brazilin (**1**) on reduction with $NaBH_4$ in MeOH followed by acid treatment. The product thus obtained was dextrorotatory, like brazilin (**1**). Sappanone B (**19**) afforded a trimethyl derivative (**20**) on methylation with CH_2N_2 or Me_2SO_4. On reduction with $NaBH_4$ in MeOH, **20** gave an about 1 : 10 mixture of **8** and **9**, both of which yielded **3** upon heating in MeOH in the presence of an acid. Moreover, the derivatives, **8** and **9**, derived from sappanol (**4**) and episappanol (**5**), respectively, afforded **20** on oxidation with CrO_3–pyridine. The structure of sappanone B is therefore (3*R*)-3,7-dihydroxy-3-(3,4-dihydroxybenzyl)chroman-4-one (**19**).

3-Deoxysappanone B (Mixture of 22 and 23)

3-Deoxysappanone B obtained from Sappan Lignum had a specific rotation of $-10.3°$ (MeOH).[4] 3-Deoxysappanone B afforded a trimethyl derivative, $[\alpha]_D$ $-3.3°$ ($CHCl_3$), on methylation with CH_2N_2 or Me_2SO_4, and a triacetyl derivative, $[\alpha]_D$ $-6.4°$ ($CHCl_3$), on acetylation. The CD curve of the trimethyl derivative of the natural product showed no maximum (Fig. 2). The acetyl derivative and 3-deoxysappanone B also exhibited similar CD curves to that of trimethyl ether. Consequently, 3-deoxysappanone B was presumed to be a mixture of enantiomers (**22** and **23**).

To verify this presumption, the levorotatory trimethyl ether (**25**) was derived from **20**. Compound **20** was mesylated with mesyl chloride in Et_3N–benzene in the presence of 4-dimethylaminopyridine to yield **21**, which was transformed into **28** with $LiAlH_4$–tetrahydrofuran (THF). Compound **28**, a mixture of the epimers at C-4, was oxidized with CrO_3–pyridine to give **25**, $[\alpha]_D$ $-50.4°$ ($CHCl_3$). The ^{1}H-NMR and mass spectra of **25** were identical with those of the trimethyl ether of 3-deoxysappanone B derived from the natural product. The CD curve of **25** thus derived from **20** showed the Cotton effects illustrated in Fig. 2.

3-Deoxysappanone B obtained as a natural product is therefore elucidated to be a mixture of the enantiomers (3*S*)-3-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-oen (**22**) and

(3*R*)-3-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-one (**23**).

3′-Deoxysappanone B (26)

The absolute stereochemistry at the C-3 position of 3′-deoxysappanone B (**26**)[3] was elucidated to be (*R*) from the interrelation with 3′-deoxysappanol (**12**),[3] which has been established to have (3*S*,4*R*)-configuration.[3] 3′-Deoxysappanone B (**26**) yielded a trimethyl derivative (**27**), $[\alpha]_D$ $-31.2°$ ($CHCl_3$), on methylation with Me_2SO_4. On treatment with CrO_3–pyridine, the trimethyl ether of 3′-deoxysappanol (**14**)[2] afforded **27**, which had a specific rotation of $-32.8°$ ($CHCl_3$). Consequently, 3′-deoxysappanone B (**26**) is (3*R*)-3,7-dihydroxy-3-(4-hydroxybenzyl)chroman-4-one.

3-Deoxysappanone B (mixture of **22** and **23**) was obtained as a mixture of enantiomers, with one (**23**) in a slight excess, because it showed a slight levorotation.

The absolute stereochemistries of homoisoflavonoids and brazilins isolated from *C. sappan* and *C. japonica* have been established in this report and our previous papers.[1,3] The probable biogenetic pathway to brazilin (**1**)[3,4,10] can thus be depicted with consideration of the stereochemistries.

The elucidation of the absolute stereochemistries of dibenzoxocin derivatives isolated from *C. sappan* and *C. japonica* is in progress.

Experimental

General——See reference 3.

The physical and spectral data for compounds **1**,[1,2] **2**,[2] **3**,[2] **4**,[2] **5**,[2] **6**,[1,4] **7**,[1,4] **8**,[2] **9**,[2] **12**,[3] **13**,[2] **14**,[2] **16**,[1] **17**,[2] **18**,[2] **19**,[1,4] **22**,[4] **23**[4] and **26**[3] have been reported.

Methylation of Phenols——Method A: A solution of CH_2N_2 in Et_2O was added to a solution of a phenol in MeOH and the mixture was allowed to stand at 4 °C for 6—24 h, then the solvents were evaporated off.

Method B: A mixture of a phenol, Me_2SO_4 (1.2—1.5 eq.) and a large excess of K_2CO_3 in dry acetone was refluxed for 2—3 h, filtered and evaporated to dryness.

If necessary, the residue was purified by silica gel column chromatography or preparative thin-layer chromatography (prep. TLC).

Acid Treatment of 4-*O*-Methylsappanol (6) and 4-*O*-Methylepisappanol (7) [Transformation into Brazilin (1)]——A solution of **6** (22 mg) in MeOH (10 ml) and conc. HCl (0.1 ml) was refluxed for 1 h. After evaporation of the solvent, the residue was purified by prep. TLC ($CHCl_3$–MeOH) to afford **1** (12 mg), $[\alpha]_D^{25}$ $+114.3°$ (c=0.40, MeOH).

Compound **7** (25 mg) was treated in a similar manner and yielded **1** (12 mg), $[\alpha]_D^{25}$ $+108.6°$ (c=0.38, MeOH). The physicochemical properties of **1** coincided with those of brazilin.[1]

Compound 10——4-*O*-Methylsappanol (**6**) (57 mg) was methylated (method A for 6 h), and the residue was separated by prep. TLC (benzene–acetone=10 : 1), yielding **10** (11 mg), $[\alpha]_D^{25}$ $+42.4°$ (c=1.18, $CHCl_3$). EI-MS *m/z*: 360 (M^+), 328, 194, 177, 168, 167, 152, 151, 137, 121. ^{1}H-NMR ($CDCl_3$) δ: 2.71 (2H, s, H-9), 3.34 (3H, s, 4-OMe), 3.66 (1H, br s, H-4), 3.77 (1H, dd, J=10.2, 1.3 Hz, H-2), 3.81, 3.84 and 3.87 (each 3H, s, OMe × 3), 4.01 (1H, d, J=10.2 Hz, H-2), 6.50 (1H, d, J=2.4 Hz, H-8), 6.53 (1H, dd, J=8.5, 2.4 Hz, H-6), 6.60—6.90 (3H, m, H-2′, 5′ and 6′), 7.04 (1H, d, J=8.5 Hz, H-5). CD (c=0.00025 mol/l, $CHCl_3$ $[\theta]^{25}$ (nm): −120 (325), +200 (297), −4780 (284), −4870 (281), +900 (250).

After 6 h, the reaction mixture showed spots of the starting material and by-products on TLC (benzene–acetone=10 : 1), and a detectable (spraying with 10% H_2SO_4 followed by heating) amount of epimer (**11**) was already present. After 16 h, a mixture of epimers was found in about 3 : 2 ratio, and the trimethyl ether of brazilin was detected. The ratio of the epimers became about 1 : 1 after 26 h. The amounts of by-products increased with increasing reaction time. When EtOH was used as a solvent, the reaction proceeded similarly, except for the formation of 4-*O*-ethyl derivatives, which were detectable after 10 h.

Compound 11——4-*O*-Methylepisappanol (**7**) (65 mg) was methylated (method A for 6 h), and the residue was separated by prep. TLC (benzene–acetone=10 : 1) to afford **11** (12 mg), $[\alpha]_D^{25}$ $-21.7°$ (c=0.92, $CHCl_3$). EI-MS *m/z*: 360 (M^+), 328, 194, 177, 168, 167, 152, 151, 137, 121. ^{1}H-NMR ($CDCl_3$) δ: 2.77 (1H, d, J=14.0 Hz, H-9), 3.04 (1H, d, J=14.0 Hz, H-9), 3.36 (3H, s, 4-OMe), 3.64 (1H, br s, H-4), 3.77 and 3.80 (3H and 6H, each s, OMe × 3), 4.23 (1H, d, J=11.0 Hz, H-2), 6.40—6.60 (2H, m, H-6 and 8), 6.80—6.96 (3H, m, H-2′, 5′ and 6′), 7.03 (1H, d, J=8.8 Hz, H-5). CD (c=0.00025 mol/l, $CHCl_3$) $[\theta]^{25}$ (nm): −740 (325), +1440 (283), +267 (280), +620 (250).

Epimerization at the C-4 position (formation of **10**) occurred as in the case of the methylation of **6**.

Acid Treatment of Compounds 10 and 11 [Transformation into 3]——A mixture of **10** (7 mg), conc. HCl (0.1 ml)

and MeOH (5 ml) was refluxed for 1 h and evaporated to dryness. The residue was purified by prep. TLC (benzene–acetone=9:1) to afford **3** (5 mg), $[\alpha]_D^{25}$ +121.3° (c=0.24, $CHCl_3$).

Compound **11** (10 mg) was treated in a similar manner, yielding **3** (8 mg), $[\alpha]_D^{25}$ +124.1° (c=0.31, $CHCl_3$).

The physicochemical properties of **3** coincided with those of the trimethyl ether of brazilin.[2]

Compound 15——A solution of **14** in 80% AcOH was heated at 100 °C for 3 h. The residue obtained after evaporation of the solvents consisted of an about 1:1 mixture of **14** and **15**, which were separated by prep. TLC (hexane–acetone=3:2) to yield pure **15**, $[\alpha]_D^{25}$ −22.5° (c=0.36, $CHCl_3$). EI-MS m/z: 316 (M^+), 177, 165, 164, 153, 152, 151, 149, 122, 121. ^{1}H-NMR ($CDCl_3$) δ: 2.75 (1H, d, J=14.0 Hz, H-9), 3.03 (1H, d, J=14.0 Hz, H-9), 3.75 and 3.80 (each 3H, s, OMe×2), 3.82 (1H, d, J=11.5 Hz, H-2), 4.11 (1H, dd, J=11.5, 1.6 Hz, H-2), 4.27 (1H, br s, H-4), 6.41 (1H, d, J=2.5 Hz, H-8), 6.51 (1H, dd, J=8.2, 2.5 Hz, H-6), 6.88 (2H, d, J=8.8 Hz, H-3′ and 5′), 7.15 (1H, d, J=8.2 Hz, H-5), 7.29 (2H, d, J=8.8 Hz, H-2′ and 6′).

Transformation of Sappanone B (19) into Brazilin (1)——Sodium borohydride (30 mg) was added to a solution of **19** (32 mg) in MeOH (10 ml) under stirring at 0 °C. After stirring at room temperature for 1 h, the reaction solution was acidified with conc. HCl and heated at 60 °C for 1 h. The mixture was evaporated to dryness and the residue was subjected to prep. TLC ($CHCl_3$–MeOH=9:1), affording **1** (13 mg), $[\alpha]_D^{25}$ +112.2° (c=0.43, MeOH). The physicochemical properties of **1** coincided with those of brazilin.[1]

Compound 20——From Sappanone B (**19**): Compound **19** was methylated (method A for 24 h and method B), and the residue was purified by silica gel column chromatography (eluting with benzene–acetone=9:1) to give **20**, $[\alpha]_D^{25}$ −31.9° (c=3.73, $CHCl_3$). EI-MS m/z: 344 (M^+), 326, 194, 193, 165, 152, 151, 137, 135, 107. ^{1}H-NMR ($CDCl_3$) δ: 2.90 (2H, s, H-9), 3.81 and 3.83 (6H and 3H, each s, OMe×3), 4.05 (1H, d, J=11.5 Hz, H-2), 4.27 (1H, d, J=11.5 Hz, H-2), 6.45 (1H, d, J=2.2 Hz, H-8), 6.63 (1H, dd, J=8.8, 2.2 Hz, H-6), 6.70—6.86 (3H, m, H-2′, 5′ and 6′), 7.79 (1H, d, J=8.8 Hz, H-5).

From Compound **8**: A solution of **8** (35 mg) in pyridine was added to CrO_3–pyridine reagent prepared from CrO_3 (58 mg) and pyridine (2 ml). The reaction solution was stirred at room temperature for 3 h, then diluted with H_2O (50 ml) and extracted with EtOAc (3×50 ml). The EtOAc extract was washed with 1 N HCl and H_2O successively, dried over Na_2SO_4 and evaporated. The residue was purified by prep. TLC (benzene–acetone=4:1) to afford **20** (32 mg), $[\alpha]_D^{25}$ −30.6° (c=1.00, $CHCl_3$).

From Compound **9**: On oxidation as above, compound **9** (23 mg) gave **20** (21 mg), $[\alpha]_D^{25}$ −32.1° (c=0.80, $CHCl_3$).

Transformation of 20 into 3——Sodium borohydride (60 mg) was added to a solution of **20** (68 mg) in EtOH (15 ml) under stirring at 0 °C. The solution was stirred at room temperature for 2 h, then diluted with H_2O and extracted with EtOAc. The EtOAc extract was washed with H_2O, dried over Na_2SO_4 and then evaporated. The residue (mixture of **8** and **9**) was separated by prep. TLC (hexane–acetone=7:3) and afforded pure **8** (5 mg) and **9** (52 mg). The physicochemical properties of **8** and **9** coincided with those of the trimethyl ethers of sappanol and episappanol, respectively.[2]

A mixture of **8** (12 mg), MeOH (10 ml) and conc. HCl (0.1 ml) was refluxed for 2 h. The solution was evaporated to dryness and the residue was purified by prep. TLC (benzene–acetone=5:1) to yield **3** (8 mg), $[\alpha]_D^{25}$ +125.3° (c=0.28, $CHCl_3$).

Compound **9** (25 mg) was treated similarly to give **3** (16 mg), $[\alpha]_D^{25}$ +120.0° (c=0.50, $CHCl_3$).

The physicochemical properties of **3** coincided with those of the trimethyl ether of brazilin.[2]

Compound 25——A mixture of **20** (75 mg), MsCl (38 mg), Et_3N (70 mg) and 4-dimethylaminopyridine (1 mg) in dry benzene (30 ml) was stirred at room temperature for 18 h. The reaction solution was worked up in a usual manner to afford **21** (85 mg). EI-MS m/z: 422 (M^+), 327, 326, 311, 295, 251, 151. ^{1}H-NMR ($CDCl_3$) δ: 3.19 (2H, s, H-9), 3.29 (3H, s, OMs), 3.84 (9H, s, OMe×3), 4.30 (1H, d, J=11.5 Hz, H-2), 4.88 (1H, d, J=11.5 Hz, H-2), 6.44 (1H, d, J=2.3 Hz, H-8), 6.65 (1H, dd, J=8.8, 2.3 Hz, H-6), 6.74—6.90 (3H, m, H-2′, 5′ and 6′), 7.86 (1H, d, J=8.8 Hz, H-5), which showed a single spot on TLC (benzene–acetone=9:1, hexane–acetone=4:1, benzene–EtOAc=7:1), and was used for the following reduction without any separation procedure.

A solution of **21** (82 mg) in dry THF (10 ml) was added to a solution of $LiAlH_4$ (20 mg) in dry THF (5 ml) under stirring at 0 °C. The mixture was stirred at room temperature for 2 h, H_2O was added, and the whole was acidified with conc. HCl, then extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried over Na_2SO_4 and evaporated to give **28** (58 mg). Compound **28** was a mixture of two epimers at the C-4 position. It was separable on TLC (benzene–acetone=7:1, hexane–acetone=7:3), but the following oxidation was performed without separation.

Compound **28** (36 mg) was treated as described for **20**, yielding **25** (25 mg), $[\alpha]_D^{25}$ −50.4° (c=0.75, $CHCl_3$). EI-MS m/z: 328 (M^+), 152, 151, 107. ^{1}H-NMR ($CDCl_3$) δ: 2.64 (1H, dd, J=12.0, 10.0 Hz, H-9), 2.80 (1H, m, H-3), 3.20 (1H, dd, J=12.0, 2.2 Hz, H-9), 3.82 and 3.86 (3H and 6H, each s, OMe×3), 4.15 (1H, dd, J=11.5, 7.5 Hz, H-2), 4.38 (1H, dd, J=11.5, 4.5 Hz, H-2), 6.39 (1H, d, J=2.2 Hz, H-8), 6.58 (1H, dd, J=8.5, 2.2 Hz, H-6), 6.70—6.88 (3H, m, H-2′, 5′ and 6′), 7.85 (1H, d, J=8.5 Hz, H-5). CD (c=0.000157 mol/l, $CHCl_3$) $[\theta]^{25}$ (nm): −3800 (332), +2170 (311), −8030 (277), +5220 (250).

Mixture of Compounds 24 and 25——3-Deoxysappanone B (mixture of **22** and **23**), $[\alpha]_D^{25}$ −10.3° (MeOH),

isolated from Sappan Lignum was methylated (methods A and B), and the residue was purified by prep. TLC (benzene–acetone = 9 : 1). The product (mixture of **24** and **25**) had a specific rotation of $-3.3°$ ($c=1.22$, $CHCl_3$). The EI-MS and ^{1}H-NMR spectra of the product were identical with those of **25** derived from **20**. The CD curve of the product is shown in Fig. 1.

Compound 27——From Compound **14**: Compound **14** (32 mg) in pyridine (2 ml) was treated as described for **20**, giving **27** (28 mg), $[\alpha]_D^{25}$ $-32.8°$ ($c=1.34$, $CHCl_3$). EI-MS m/z: 314 (M^+), 296, 194, 193, 165, 151, 122, 121. ^{1}H-NMR ($CDCl_3$) δ: 2.92 (2H, s, H-9), 3.78 and 3.87 (each 3H, s, OMe × 2), 4.06 (1H, d, $J=11.5$ Hz, H-2), 4.28 (1H, d, $J=11.5$ Hz, H-2), 6.48 (1H, d, $J=2.2$ Hz, H-8), 6.67 (1H, dd, $J=8.8$, 2.2 Hz, H-6), 6.84 (2H, d, $J=8.5$ Hz, H-3′ and 5′), 7.14 (2H, d, $J=8.5$ Hz, H-2′ and 6′), 7.83 (1H, d, $J=8.8$ Hz, H-5).

From 3′-Deoxysappanone B (**26**): Compound **26** (8 mg) was methylated (method B) and the residue was purified by prep. TLC (benzene–acetone = 9 : 1) to afford **27** (6 mg), $[\alpha]_D^{25}$ $-31.2°$ ($c=0.23$, $CHCl_3$). The physicochemical properries of **27** were identical with those of **27** derived from **14**.

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Total Synthesis of Neolignans, Americanin A and Isoamericanin A

HITOSHI TANAKA, ICHIRO KATO, and KAZUO ITO*

Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, Japan

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The condensation reaction of 3-benzyloxy-4-hydroxybenzaldehyde with 2,3-epoxy-3-[(3,4-dimethoxymethoxy)phenyl]-1-propanol, prepared from caffeic acid in four steps, afforded the ether (**11**) in good yield. Mesylation of **11** followed by treatment with potassium carbonate, provided the epoxide (**13**), which was converted to the debenzylation product (**14**) by hydrogenolysis. Compound **14** underwent cyclization with potassium carbonate to yield the *trans* dioxane derivative (**15**). Reaction of **15** with the ylide (**16**) followed by hydrolysis furnished americanin A (**1**). Isoamericanin A (**3**) was similarly synthesized from another condensation product (**18**).

Keywords——Phytolaccaceae; *Phytolacca americana*; americanin A; isoamericanin A; regioisomer; benzodioxane; epoxide; neolignan; antihepatotoxic activity

The neolignan americanin A (**1**) has been isolated[1a)] from the seeds of *Phytolacca americana* (Phytolaccaceae) together with americanin B and americanin D. Americanin A is a racemic compound (no optical activity),[1b)] and shows antihepatotoxic activity.[1c)] The structure of americanin A was first represented[1a,c)] as the formula (**3**), but recently the revised structure (**1**) was proposed[1b)] on the basis of a degradation study of its dimethylether.

Here we describe the synthetic confirmation of the structures of americanin A (**1**) and its regioisomer (isoamericanin A)[2)] (**3**), in which a new dioxane formation[3-6)] by treatment of compounds **14** and **21** with potassium carbonate was utilized.

The key intermediate (**14**) for the synthesis of **1** was prepared as follows. Caffeic acid (**5**) was converted to a methoxymethyl (MOM) ether (**7**) by esterification with acidic methanol followed by treatment with chloromethoxymethane. Reduction of **7** with lithium aluminum hydride gave the alcohol (**8**) in 78% yield, and this was transformed into an epoxide (**9**) in 84% yield by oxidation with *tert*-butyl hydroperoxide in the presence of vanadyl acetylacetonate. Condensation of **9** with 3-benzyloxy-4-hydroxybenzaldehyde (**10**) in the presence of sodium hydroxide gave the ether (**11**) as a stereochemically homogeneous product in good yield. The configuration (*erythro* or *threo*) of the ether (**11**) was ambiguous at this stage, but the following transformation (see below) of **11** to the *trans* dioxane (**15**) established its configuration as *erythro*. Mesylation of **11** in the usual manner gave the corresponding mesylate (**12**) in 72% yield, and subsequent treatment with potassium carbonate provided an epoxide (**13**) in an excellent yield. Debenzylation of **13** by hydrogenolysis yielded the first target substance (**14**).

Compound **14** underwent cyclization with potassium carbonate to yield the dioxane derivative (**15**) in 86% yield. The mass spectrum (MS) of **15** showed the characteristic peak at m/z 254 due to a retro Diels–Alder fragmentation of the benzodioxane moiety, and the proton nuclear magnetic resonance (^{1}H-NMR) spectrum revealed a doublet signal of H-7 at δ 5.01 with a coupling constant ($J=8$ Hz) typical of *trans* orientation of the benzodioxane ring.

Next, we investigated the Wittig synthesis of the α,β-unsaturated aldehyde moiety in americanin A. The attempted Wittig reaction of **15** with the ylide prepared from 1,3-dioxan-2-ylmethyltriphenylphosphonium bromide[7)] or diethyl formylmethylphosphonate diethylacetal,[8)] was unsuccessful, the starting material (**15**) being recovered unchanged. However, the

1: R=H
2: R=MOM

5: $R_1=R_2=H$
6: $R_1=H$, $R_2=CH_3$
7: $R_1=MOM$, $R_2=CH_3$

8

9

10

3: R=H
4: R=MOM

11: R=H
12: R=Ms

13: R=Bzl
14: R=H

15

16

17

18: R=H
19: R=Ms

20: R=Bzl
21: R=H

22

$MOM=CH_2OCH_3$

Chart 1

ylide (**16**)[9] prepared from diethyl 2-(cyclohexylimino)ethylphosphonate by treatment with sodium hydride, when reacted with **15** at room temperature for 1 h, yielded the desired α,β-unsaturated aldehyde (**2**). Acid hydrolysis of **2** gave americanin A (**1**), which was identical with the natural sample[1] on the basis of mixed melting point determination and direct comparison of infrared (IR) spectra.

We also attempted the synthesis of isoamericanin A (**3**) in a similar manner. Compound **18** was prepared by condensation of **9** with 4-benzyloxy-3-hydroxybenzaldehyde (**17**) under basic conditions in 80% yield. Mesylation of **18** followed by epoxidation and subsequent debenzylation, as described for **14**, afforded the epoxide (**21**) in 71% overall yield from **18**. Treatment of the epoxide (**21**) with potassium carbonate furnished the benzodioxane (**22**), in which the substituents on the benzodioxane nucleus are disposed in a *trans* orientation. Finally, **22** was reacted with the ylide (**16**) to yield the α,β-unsaturated aldehyde (**4**) which was converted to isoamericanin A (**3**) by acid treatment. The above syntheses of americanin A and isoamericanin A established that the correct structure for americanin A is represented by the formula (**1**).

Experimental

All melting points are uncorrected. Column chromatography was run on Merck silica gel 60 (70—230 mesh). Thin-layer chromatography (TLC) was performed on glass plates precoated with Kieselgel 60 F_{254} (Merck). MS were recorded on a Hitachi M-52 spectrometer and high-resolution MS and secondary ion MS (SIMS) on a Hitachi M-80 spectrometer. IR spectra were obtained on a JASCO IRA-3 spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-PS-100 nuclear magnetic resonance spectrometer and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra on a JEOL JNM-FX-100, with tetramethylsilane as an internal standard. Chemical shifts are quoted in parts per million (s = singlet, d = doublet, dd = doublet-of-doublets, t = triplet, q = quartet, m = multiplet, br = broad).

Methyl 3-(3,4-Dihydroxy)phenyl-2-propenoate (6)——A mixture of caffeic acid (**5**) (25 g) in conc. H_2SO_4 (1.3 ml) and MeOH (250 ml) was refluxed for 8 h. The organic solvent was removed *in vacuo*. The resulting precipitate was collected and washed with water. The crude product was recrystallized from MeOH to give **6** as colorless needles (26 g, 96%). mp 152—153 °C (lit.,[10] mp 152 °C).

Methyl 3-(3,4-Dimethoxymethoxy)phenyl-2-propenoate (7)——NaH (60% in mineral oil) (9.6 g), washed with dry ether, was suspended in dry tetrahydrofuran (THF) (100 ml). The slurry was cooled to 0 °C under a nitrogen atmopshere and a solution of **6** (26 g) in dry THF (100 ml) was slowly added. After the addition, the mixture was stirred at room temperature for 1 h. Then the mixture was cooled to 0 °C and a solution of chloromethoxymethane (30.5 ml) in dry THF (50 ml) was added dropwise. The mixture was stirred at room temperature for 8 h, then quenched by adding water (50 ml). The resulting mixture was extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (20 : 1), giving **7** as a colorless oil (34 g, 90%). High-resolution MS *m/z*: 282.1102. Calcd for $C_{14}H_{18}O_6$ (M^+). Found: 282.1116. MS *m/z*: 282 (M^+), 207, 206 (100%), 175, 146, 145. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1705, 1640, 1600, 1585. ^{1}H-NMR ($CDCl_3$) δ: 3.47, 3.49, 3.75 (9H, 3 × s, 3 × OCH_3), 5.25 (4H, s, 2 × OCH_2OCH_3), 6.35 (1H, d, J = 16 Hz, C_2-H), 7.14 (3H, m, 3 × aromatic protons), 7.63 (1H, d, J = 16 Hz, C_3-H).

3-(3,4-Dimethoxymethoxy)phenyl-2-propen-1-ol (8)——A suspension of lithium aluminum hydride (4.0 g) in dry ether was added dropwise to a solution of **7** (30 g) in dry ether (30 ml) and dry THF (30 ml) at −10 °C. After the addition, AcOEt (50 ml) and then water (30 ml) were added to the reaction mixture. The resulting mixture was filtered and the filtrate was concentrated. The residue was chromatographed on a silica gel column with a mixture of benzene and AcOEt (1 : 1), giving **8** as a colorless oil (21 g, 78%). High-resolution MS *m/z*: 254.1153 Calcd for $C_{13}H_{18}O_5$ (M^+). Found: 254.1164. MS *m/z*: 254 (M^+), 178 (100%), 162, 161, 150, 135, 122, 120, 119. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1580, ^{1}H-NMR ($CDCl_3$) δ: 2.08 (1H, br s, OH), 3.56 (6H, s, 2 × OCH_3), 4.33 (2H, d, J = 5 Hz, CH_2OH), 5.28 (4H, s, 2 × OCH_2OCH_3), 6.31 (1H, m, C_2-H), 6.64 (1H, d, J = 16 Hz, C_3-H), 7.00—7.40 (3H, m, 3 × aromatic protons).

2,3-Epoxy-3-[(3,4-dimethoxymethoxy)phenyl]-1-propanol (9)——A solution of *tert*-butyl hydroperoxide (70% in water) (8.21 ml) in CH_2Cl_2 (50 ml) was added to a mixture of vanadyl acetylacetonate (40 mg) and **8** (7.62 g) in CH_2Cl_2 (100 ml) and the reaction mixture was stirred at room temperature for 6 h. The organic solvent was evaporated off. The residue was chromatographed on a silica gel column with a mixture of benzene and AcOEt (1 : 1), giving **9** as a colorless oil (6.80 g, 84%). High-resolution MS *m/z*: 270.1102 Calcd for $C_{13}H_{18}O_6$ (M^+). Found: 270.1096. MS *m/z*: 270 (M^+), 256, 194, 180, 151, 149, 136, 135 (100%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500, 1580. ^{1}H-NMR ($CDCl_3$) δ: 2.67 (1H, br s, OH), 3.44—4.00 (4H, m, C_1-H, C_2-H, and C_3-H), 3.57 (6H, s, 2 × OCH_3), 5.28 (4H, s, 2 × OCH_2OCH_3), 6.80—7.32 (3H, m, 3 × aromatic protons).

Condensation of 9 with 10 (Formation of 11)——A solution of **10**[11] (6.83 g) in 1% aq NaOH (120 ml) was stirred at 70 °C under a nitrogen atmosphere and excess epoxide (**9**) (9.0 g) was added over 10 min at 70 °C. The mixture was stirred at the same temperature for 2.5 h and then poured into 1 N NaOH. After extraction with CH_2Cl_2, the organic layer was washed successively with 1 N NaOH and brine, dried over Na_2SO_4, and concentrated. The residue was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **11** as a colorless oil (12.0 g, 81% based on **10** consumed). SIMS *m/z*: 499 (M^++1), 421, 271, 239. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1685, 1600. ^1H-NMR ($CDCl_3$) δ: 3.31—3.95 (3H, m, C_8-H and C_9-H), 3.41, 3.46 (6H, 2×s, 2×OCH_3), 5.13 (4H, s, 2×OCH_2), 5.14 (2H, s, OCH_2), 5.27 (1H, d, J=6 Hz, C_7-H), 6.65—7.51 (6H, m, 6×aromatic protons), 9.66 (1H, s, CHO).

Mesylation of 11 (Formation of 12)——A solution of MsCl (0.31 ml) in CH_2Cl_2 (2 ml) was added to a solution of **11** (1.81 g) in pyridine (1 ml) at −10 °C. The mixture was stirred at the same temperature for 6 h, then the reaction mixture was poured into ice-water and extracted with $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **12** as a colorless oil (1.5 g, 72%). SIMS *m/z*: 577 (M^++1), 461, 349, 277. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1690, 1600. ^1H-NMR ($CDCl_3$) δ: 2.84 (3H, s, SO_2CH_3), 3.39, 3.43 (6H, 2×s, 2×OCH_3) 4.17 (1H, m, C_8-H), 4.40 (2H, d, J=4 Hz, C_9-H), 5.07 (2H, s, OCH_2), 5.15 (4H, s, 2×OCH_2), 5.16 (1H, d, J=6 Hz, C_7-H), 6.70—7.66 (6H, m, 6×aromatic protons), 9.62 (1H, s, CHO).

Epoxidation of 12 (Formation of 13)——A mixture of **12** (1.13 g) and anhydrous K_2CO_3 (270 mg) in MeOH (5 ml) was stirred at room temperature for 30 min. The reaction mixture was filtered, then the filtrate was evaporated to dryness, and the residue was dissolved in $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **13** as a colorless oil (930 mg, 99%). $C_{27}H_{28}O_8$. MS *m/z*: 480 (M^+), 450, 437, 349, 253, 228, 221, 209, 189, 177, 149, 147, 135, 119. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1685, 1600, 1585. ^1H-NMR ($CDCl_3$) δ: 2.62—2.95 (2H, m, C_9-H), 3.22—3.54 (1H, m, C_8-H), 3.42, 3.46 (6H, 2×s, 2×OCH_3), 5.10, 5.13, 5.14 (6H, 3×s, 3×OCH_2), 5.20 (1H, d, J=2 Hz, C_7-H), 6.78—7.50 (6H, m, 6×aromatic protons), 9.68 (1H, s, CHO).

Debenzylation of 13 (Formation of 14)——A solution of **13** (923 mg) in AcOEt (20 ml) was hydrogenated over 5% Pd–C (100 mg) under an H_2 atmosphere. The reaction mixture was filtered and the filtrate was concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **14** as a colorless oil (710 mg, 95%). High-resolution MS *m/z*: 390.1313 Calcd for $C_{20}H_{22}O_8$ (M^+). Found: 390.1350. MS *m/z*: 390 (M^+), 254 (100%), 177, 149, 147, 135, 119. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1680, 1600. ^1H-NMR ($CDCl_3$) δ: 2.72—3.42 (3H, m, C_8-H and C_9-H), 3.46 (6H, s, 2×OCH_3), 4.90 (1H, d, J=2 Hz, C_7-H), 5.21 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.66—7.50 (6H, m, 6×aromatic protons), 9.62 (1H, s, CHO).

Cyclization of 14 (Formation of 15)——A mixture of **14** (675 mg) and anhydrous K_2CO_3 (230 mg) in MeOH (10 ml) was stirred at room temperature for 30 min. The reaction mixture was filtered, then the filtrate was evaporated to dryness, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with water, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **15** as a colorless oil (580 mg, 86%). High-resolution MS *m/z*: 390.1313 Calcd for $C_{20}H_{22}O_8$ (M^+). Found: 390.1290. MS *m/z*: 390 (M^+, 100%), 314, 296, 254, 253, 178, 177, 147, 136, 135. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1690, 1605, 1585. ^1H-NMR ($CDCl_3$) δ: 3.43—3.93 (2H, m, C_9-H), 3.49 (6H, s, 2×OCH_3), 4.01 (1H, m, C_8-H), 5.01 (1H, d, J=8 Hz, C_7-H), 5.21 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.91—7.49 (6H, m, 6×aromatic protons), 9.77 (1H, s, CHO).

Wittig Reaction of 15 (Formation of 2)——A solution of diethyl 2-(cyclohexylimino)ethylphosphonate[9] (430 mg) in dry THF (5 ml) was added to a suspension of dry ether-washed NaH (60% in mineral oil) (40 mg) in dry THF (5 ml) with stirring and ice-cooling under a nitrogen atmosphere, and the mixture was stirred for 1 h. A solution of **15** (320 mg) in dry THF (5 ml) was added, and the whole was stirred at room temperature for 1 h. The reaction mixture was poured into ice-water and extracted with CH_2Cl_2. The organic layer was washed with saturated NaCl, dried over Na_2SO_4, and concentrated. The crude product was chromatographed on a silica gel column with a mixture of $CHCl_3$ and acetone (5:1), giving **2** as a colorless oil (290 mg, 85%). High-resolution MS *m/z*: 416.1470 Calcd for $C_{22}H_{24}O_8$ (M^+). Found: 416.1461. MS *m/z*: 416 (M^+, 100%), 340, 322, 281, 254, 251, 178, 160, 150, 136. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1670, 1620, 1610, 1580. ^1H-NMR ($CDCl_3$) δ: 3.48—3.92 (2H, m, C_9-H), 3.50 (6H, s, 2×OCH_3), 4.04 (1H, m, C_8-H), 4.99 (1H, d, J=8 Hz, C_7-H), 5.22 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.54 (1H, dd, J=16, 8 Hz, $C_{8'}$-H), 6.88—7.24 (6H, m, 6×aromatic protons), 7.32 (1H, d, J=16 Hz, $C_{7'}$-H), 9.59 (1H, d, J=8 Hz, CHO).

Americanin A (1)——A mixture of **2** (85 mg), 2 N HCl (1 ml) and MeOH (1 ml) was stirred at room temperature for 2 h. The reaction mixture was poured into ice-water and extracted with AcOEt. The organic layer was washed with water, dried over Na_2SO_4, and concentrated. The crude product was recrystallized from MeOH to give **1** as colorless needles (51 mg, 76%). mp 245 °C (lit.,[1] mp 246—247 °C). High-resolution MS *m/z*: 328.0946 Calcd for $C_{18}H_{16}O_6$ (M^+). Found: 328.0924. MS *m/z*: 328 (M^+), 166, 164, 123, 110. IR ν_{max}^{KBr} cm^{-1}: 3300, 1645, 1605, 1575. ^1H-NMR (DMSO-d_6) δ: 3.11—3.71 (2H, m, C_9-H), 4.07 (1H, m, C_8-H), 4.87 (1H, d, J=8 Hz, C_7-H), 6.49—7.35 (6H, m, 6×aromatic protons), 6.84 (1H, dd, J=16, 8 Hz, $C_{8'}$-H), 7.57 (1H, d, J=16 Hz, $C_{7'}$-H), 8.95 (2H, br s, 2×OH), 9.53 (1H, d, J=8 Hz, CHO). ^{13}C-NMR (DMSO-d_6) δ: 194.0 (d, C-9′), 153.1 (d, C-8′), 146.5 (s, C-3′), 145.9 (s, C-4), 145.2 (s, C-3), 143.5 (s, C-4′), 127.5 (s, C-1′), 127.1 (s, C-1), 126.8 (d, C-7′), 122.6 (d, C-6′), 118.8 (d, C-6), 117.3 (d, C-5′), 116.7 (d, C-2′), 115.5 (d, C-5), 114.9 (d, C-2), 78.1 (d, C-8), 76.1 (d, C-7), 60.0 (t, C-9).

Condensation of 9 with 17 (Formation on 18)——A mixture of **17**[11] (4.2 g) and NaOH (736 mg) in water (74 ml) was stirred at 70 °C under a nitrogen atmosphere and excess epoxide (**9**) (6.7 g) was added over 10 min. The mixture was stirred at the same temperature for 2.5 h. The reaction mixture was treated in a manner similar to that descirbed for **11** to give **18** as a colorless oil (7.3 g, 80%; based on **17** consumed). SIMS *m/z*: 499 ($M^+ + 1$), 421, 271, 239. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1685, 1600. ^{1}H-NMR ($CDCl_3$) δ: 2.81, 3.14 (2H, 2×brs, 2×OH), 3.38, 3.41 (6H, 2×s, 2×OCH_3), 3.56—4.00 (3H, m, C_8-H and C_9-H), 5.11 (4H, s, 2×OCH_2), 5.13 (2H, s, OCH_2), 5.21 (1H, d, J=6 Hz, C_7-H), 6.82—7.40 (6H, m, 6×aromatic protons), 9.56 (1H, s, CHO).

Mesylation of 18 (Formation of 19)——A solution of MsCl (1.11 ml) in CH_2Cl_2 (2 ml) was added dropwise to a mixture of **18** (6.5 g), CH_2Cl_2 (4 ml) and pyridine (3 ml) at −10 °C. The mixture was stirred at the same temperature for 6 h, then treated in a manner similar to that described for **12** to give **19** as a colorless oil (5.5 g, 73%). SIMS *m/z*: 577 ($M^+ + 1$), 461, 349, 277, 221. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550, 1685, 1600, 1355, 1170. ^{1}H-NMR ($CDCl_3$) δ: 2.88 (3H, s, SO_2CH_3), 3.42, 3.44 (6H, 2×s, 2×OCH_3), 4.16 (1H, m, C_8-H), 4.40 (2H, d, J=4 Hz, C_9-H), 5.12 (1H, d, J=6 Hz, C_7-H), 5.14 (6H, s, 3×OCH_2), 6.84—7.44 (6H, m, 6×aromatic protons), 9.60 (1H, s, CHO).

Epoxidation of 19 (Formation of 20)——A mixture of **19** (5.2 g) and anhydrous K_2CO_3 (1.25 g) in MeOH (20 ml) was stirred at room temperature for 30 min, then treated in manner similar to that described for **13** to give **20** as a colorless oil (4.3 g, 99%), $C_{27}H_{28}O_8$. MS *m/z*: 480 (M^+), 437, 393, 349, 317, 285, 253, 221, 209, 189, 177, 149, 147, 135, 119. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1685, 1600. ^{1}H-NMR ($CDCl_3$) δ: 2.60—2.82 (2H, m, C_9-H), 3.10—3.40 (1H, m, C_8-H), 3.44 (6H, s, 2×OCH_3), 5.14 (1H, d, J=2 Hz, C_7-H), 5.16 (6H, s, 3×OCH_2), 6.84—7.44 (6H, m, 6×aromatic protons), 9.62 (1H, s, CHO).

Debenzylation of 20 (Formation of 21)——A solution of **20** (4.14 g) in AcOEt (50 ml) was hydrogenated over 5% Pd–C (400 mg) under an H_2 atmosphere, then treated in a manner similar to that described for **14** to give **21** as a colorless oil (3.3 g, 98%). High-resolution MS *m/z*: 390.1313 Calcd for $C_{20}H_{22}O_8$ (M^+). Found: 390.1329. MS *m/z*: 390 (M^+), 254 (100%), 177, 149, 147, 135, 119. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3530, 1680, 1600. ^{1}H-NMR ($CDCl_3$) δ: 2.86—3.01 (1H, m, C_9-H), 3.15—3.27 (1H, m, C_9-H), 3.29—3.43 (1H, m, C_8-H), 3.51 (6H, s, 2×OCH_3), 4.98 (1H, d, J=2 Hz, C_7-H), 5.23 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.91—7.53 (6H, m, 6×aromatic protons), 9.65 (1H, s, CHO).

Cyclization of 21 (Formation of 22)——A mixture of **21** (3.3 g) and anhydrous K_2CO_3 (1.2 g) in MeOH (90 ml) was stirred at room temperature for 30 min, then treated in a manner similar to that described for **15** to give **22** as a colorless oil (2.8 g, 85%). High-resolution MS *m/z*: 390.1313 Calcd for $C_{20}H_{22}O_8$ (M^+). Found: 390.1288. MS *m/z*: 390 (M^+, 100%), 314, 296, 254, 253, 178, 177, 147, 136, 135. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1690, 1605, 1585. ^{1}H-NMR ($CDCl_3$) δ: 1.82 (1H, brs, OH), 3.50 (6H, s, 2×OCH_3), 3.55 (1H, dd, J=12.5, 3 Hz, C_9-H), 3.83 (1H, dd, J=12.5, 3 Hz, C_9-H), 4.08 (1H, m, C_8-H), 4.94 (1H, d, J=8 Hz, C_7-H), 5.20 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.92—7.40 (6H, m, 6×aromatic protons), 9.78 (1H, s, CHO).

Wittig Reaction of 22 (Formation of 4)——A solution of diethyl 2-(cyclohexylimino)ethylphosphonate[9] (2.38 g) in dry THF (10 ml) was added to a suspension of dry ether-washed NaH (60% in mineral oil) (210 mg) in dry THF (10 ml) with stirring and ice-cooling under a nitrogen atmosphere, and the mixture was stirred for 1 h. A solution of **22** (1.77 g) in dry THF (10 ml) was added, and the whole was stirred at room temperature for 1 h. The reaction mixture was treated in a manner similar to that described for **2** to give **4** as a colorless oil (1.56 g, 83%). High-resolution MS *m/z*: 416.1470 Calcd for $C_{22}H_{24}O_8$ (M^+). Found: 416.1433. MS *m/z*: 416 (M^+, 100%), 340, 322, 281, 254, 251, 178, 160, 150, 136. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1670, 1620, 1610, 1580. ^{1}H-NMR ($CDCl_3$) δ: 1.98 (1H, brs, OH), 3.44—3.92 (2H, m, C_9-H), 3.48 (6H, s, 2×OCH_3), 4.05 (1H, m, C_8-H), 4.92 (1H, d, J=8 Hz, C_7-H), 5.20 (4H, s, 2×$OC\underline{H}_2OCH_3$), 6.50 (1H, dd, J=16, 8 Hz, $C_{8'}$-H), 6.86—7.16 (6H, m, 6×aromatic protons), 7.29 (1H, d, J=16 Hz, $C_{7'}$-H), 9.56 (1H, d, J=8 Hz, CHO).

Isoamericanin A (3)——A mixture of **4** (492 mg), 2 N HCl (5 ml) and MeOH (5 ml) was stirred at room temperature for 2 h. The reaction mixture was treated in a manner similar to that described for **1** to give a solid, which was recrystallized from MeOH to give **3** as yellow needles (320 mg, 82%), mp 174—176 °C (lit.,[2] mp 177—178 °C). High-resolution MS *m/z*: 328.0946 Calcd for $C_{18}H_{16}O_6$ (M^+). Found: 328.0938. MS *m/z*: 328 (M^+), 166, 164, 123, 110. IR ν_{max}^{KBr} cm^{-1}: 3200, 1650, 1600, 1575. ^{1}H-NMR (DMSO-d_6) δ: 3.16—3.72 (2H, m, C_9-H), 4.14 (1H, m, C_8-H), 4.85 (1H, d, J=8 Hz, C_7-H), 6.66 (1H, dd, J=16, 8 Hz, $C_{8'}$-H), 6.68—7.36 (6H, m, 6×aromatic protons), 7.52 (1H, d, J=16 Hz, $C_{7'}$-H), 9.02 (1H, brs, OH). 9.52 (1H, d, J=8 Hz, CHO). ^{13}C-NMR (DMSO-d_6) δ: 194.0 (d, C-9′), 153.1 (d, C-8′), 146.3 (s, C-3′), 145.9 (s, C-4), 145.3 (s, C-3), 143.9 (s, C-4′), 127.4 (s, C-1′), 127.3 (s, C-1), 126.8 (d, C-7′), 123.0 (d, C-6′), 118.9 (d, C-6), 117.3 (d, C-5′), 116.8 (d, C-2′), 115.6 (d, C-5), 115.1 (d, C-2), 78.8 (d, C-8), 75.7 (d, C-7), 60.2 (t, C-9).

The IR, ^{1}H-NMR, and ^{13}C-NMR spectra of the product were superimposable on those of a natural specimen.[2]

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Studies on Sialic Acids. VIII. The 1,4-Lactone Derivatives of *N*-Acetylneuraminic Acid

KIMIO FURUHATA,[a] SHINGO SATO,[a] KATSUKO ANAZAWA,[a] MOTOAKI GOTO,[b] HIROAKI TAKAYANAGI,[a] and HARUO OGURA*,[a]

School of Pharmaceutical Sciences, Kitasato University,[a] Shirokane, Minato-ku, Tokyo 108, Japan, and Central Research Laboratory, MECT Co., Ltd.,[b] Nishishinjuku, Shinjuku-ku, Tokyo 163, Japan

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The reactions of *N*-acetylneuraminic acid with benzyl, allyl, and methyl halides in the presence of excess cesium carbonate gave the corresponding *N*-acetyl-2-*O*-alkyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-noneno-1,4-lactones in good yields. The structures and stereochemistry of these 1,4-lactonic sialic acid derivatives were elucidated from the infrared and proton nuclear magnetic resonance spectra and by X-ray crystal analysis.

Keywords——*N*-acetylneuraminic acid; ^{1}H-NMR; X-ray crystal analysis; 1,4-lactone; sialic acid; cesium carbonate

The sialic acids are biologically important and are widely distributed in nature in various forms. Recently, new types of sialic acids have been isolated from the lipopolysaccharides of *Pseudomonas aeruginosa* and *Shigella boydii*,[1] and from egg polysialoglycoprotein of *Salmo gairdneri*.[2] We have been examining the synthesis of various lactone derivatives of *N*-acetylneuraminic acid with the aim of developing new strategies for the synthesis of various derivatives of sialic acid.

Several syntheses of 1,4-lactone derivatives of *N*-acetylneuraminic acid (1) have been reported. *N*-Acetylneuramino-1,4-lactone diethyldithioacetal (5-acetamido-2,2-bis(ethylthio)-2,3,5-trideoxy-D-*glycero*-D-*galacto*-nonulosono-1,4-lactone) was synthesized by Kuhn and Brossmer,[3] and the structure was confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopic techniques by Robien *et al.*[4] The synthesis of 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone has been reported, but without full details.[5,6] In this report, we describe a new and efficient synthesis of 1,4-lactone derivatives of **1**. We also describe the determination of the molecular structure of 5-acetamido-6,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-*O*-methyl-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (**6**) by X-ray crystal analysis. Benzyl esterification of the cesium salt of **1** was carried out with benzyl bromide in *N*,*N*-dimethylformamide (DMF). The reaction was completed in 2 h at room temperature to give benzyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**2**) as a crystalline product from 2-propanol in 80% yield. Treatment of **2** with an excess amount of cesium carbonate and benzyl bromide in DMF for 2 h at 40 °C gave 5-acetamido-2-*O*-benzyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (**3**) in 80% yield after recrystallization. Furthermore, we have found a facile method for the synthesis of **3** by the treatment of **1** with an excess amount of cesium carbonate and benzyl bromide. Thin-layer chromatography (TLC) of the reaction mixture showed three main spots of **3**, the starting material (**1**), and benzyl bromide, together with a few trace spots. This result suggests that this lactonization for **1** is highly regioselective. The reaction mechanism is considered to involve intramolecular

Chart 1

TABLE I. ^{1}H-NMR Spectral Data

Proton	Compound			
	3	4	5	6
3-H	6.408 d (2.0)	6.287 d (2.0)	6.287 d (1.8)	5.974 d (2.2)
4-H	5.436 t (2.0)	5.407 t (2.0)	5.420 t (1.8)	4.915 t (2.2)
5-H	3.830 ddd (2.0, 9.5, 10.5)	4.200 ddd (2.0, 9.4, 10.2)	4.212 dt (1.8, 10.5)	4.529 dt (2.2, 9.9)
6-H	3.829 dd (10.5, 8.0)	3.810 dd (10.2, 8.2)	3.831 dd (10.5, 8.1)	5.513 dd (2.2, 9.9)
7-H	3.232 dd (8.0, 7.3)	3.233 dd (8.5, 7.5)	3.238 dd (8.7, 7.3)	5.407 dd (8.8, 2.2)
8-H	3.498 dddd (8.0, 3.0, 5.8, 5.2)	3.487 dddd (8.5, 3.3, 6.0, 5.2)	3.500 dddd (8.7, 3.3, 6.0, 5.5)	5.090 ddd (8.8, 2.9, 5.5)
9-H	3.608 ddd (3.0, −10.8, 5.8)	3.600 ddd (3.3, −11.0, 5.2)	3.610 ddd (3.3, −10.9, 5.9)	4.248 dd (2.9, −12.5)
9-H′	3.370 ddd (3.0, −10.8, 5.8)	3.380 ddd (6.0, −11.0, 5.2)	3.398 ddd (6.0, −10.9, 5.9)	4.012 dd (5.5, −12.5)
NH	7.745 d (9.5)	7.738 d (9.4)	7.735 d (9.5)	5.912 d (9.9)
6-OH	4.708 d (8.0)	4.705 d (8.2)	4.700 d (8.1)	
7-OH	4.288 d (7.3)	4.287 d (7.5)	4.285 d (7.3)	
8-OH	3.498 d (5.2)	4.468 d (5.2)	4.458 d (5.5)	
9-OH	4.370 t (5.8)	4.375 t (5.2)	4.362 t (5.9)	
NAc	1.715 s	1.730 s	1.745 s	1.874 s
Others	4.875 (1H, d, J= 12.0 Hz, -CHph) 4.992 (1H, d, J= 12.0 Hz, -CHph) 7.390 (5H, s, ph)	4.4 (2H, m, allyl 1′-H) 5.30 (2H, m, allyl 3′-H) 5.956 (1H, m, allyl, 2′-H)	3.737 (3H, s, 3-OMe)	2.045 (3H, s, OAc) 2.077 (3H, s, OAc) 2.104 (3H, s, OAc) 2.151 (3H, s, OAc) 3.737 (3H, s, 3-OMe)

δ values in DMSO-d_6 at 19 °C and coupling constants in Hz.

lactonization, as shown in Chart 1.

The structure of 3 was established by comparison of the spectral data for 2 and 3. The field desorption-mass spectrum (FD-MS) of 3 showed the same principal ion as that of 2 at m/z 382 ($M^+ + 1$) and the infrared (IR) absorptions at $1710\,cm^{-1}$ for 2 and $1765\,cm^{-1}$ for 3 can be assigned to an ester C=O stretching bond and a conjugated lactone C=O stretching bond, respectively.

All the signals in the ^{1}H-NMR spectrum of 3 were assigned by homonuclear double-resonance studies, and the chemical shifts and coupling constants are summarized in Table I. The major differences between 2 and 3 were found to be the chemical shifts and coupling constants of protons on C-3 and C-4. Comparison of the ^{1}H-NMR spectra of 2 and 3 indicated that they differed by the presence of an olefinic proton at δ_H 6.41 (1H, d, $J_{3,4}$ = 2.0 Hz) in 3 instead of the C-3 methylene protons in 2. The value of $J_{3,4}$ = 2.0 Hz of 3 suggests a five- or six-membered ring system. The resonance of H-4 in 3 is readily identified because of its characteristic shift range at the lowest field as a methine proton. These results clearly identified the structure of 3.

In the same manner, treatment of 1 in DMF with allyl bromide or methyl iodide in the presence of an excess amount of cesium carbonate gave the corresponding 1,4-lactone compounds 4 and 5, respectively, in 70—80% yield. These structures were supported by the ^{1}H-NMR spectra as shown in Table I.

We attempted to confirm the 1,4-lactone structure of 6 by means of X-ray crystal structure analysis. Good crystals of 5-acetamido-6,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-methyl-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (6) for X-ray study were prepared by recrystallization from acetone solution. A crystal with the dimensions of $0.3 \times 0.4 \times 0.4\,mm^3$ was used for the structure determination. The cell dimensions and diffraction intensities were measured on a Rigaku four-circle diffractometer, using graphite-monochromated CuK_α radiation.

Crystal data: $C_{20}H_{27}NO_{12}$, monoclinic, space group $P2_1$, a=13.721(2), b=8.839(1), c= 9.548(1) Å, β=91.05(2)°, V=1157.9 Å^3, Z=2, D_c=1.357 g·cm^{-1}, D_o=1.36 g·cm^{-3}. Two thousand seven hundred and fifty independent reflections in the range of $2\theta < 135°$ were collected by the use of the 2θ–ω scan mode with a scanning rate of 4° (2θ) min^{-1}. A total of 2189 independent reflections with $|F_o| > 3\sigma(|F_o|)$ were obtained and corrected for Lorentz and polarization factors but not for absorption. The structure was solved by a direct method using

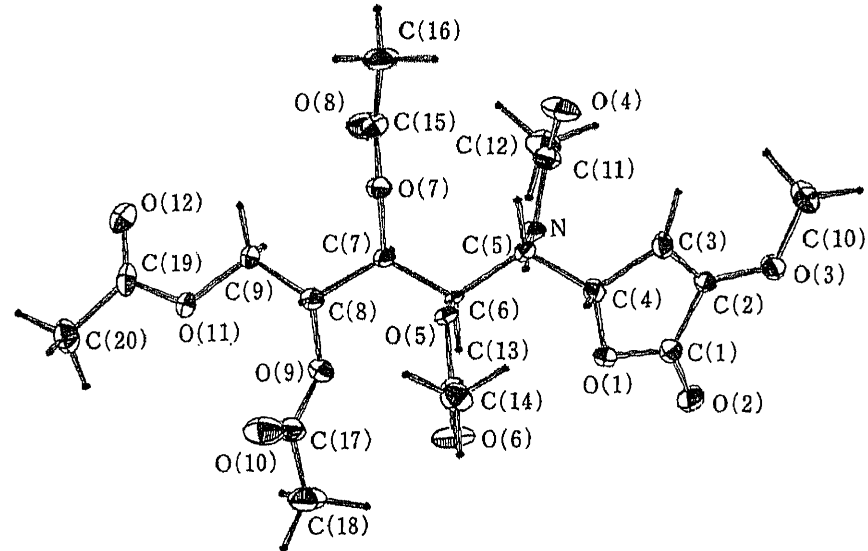

Fig. 1. A Perspective View of 6 and the Atomic Numbering

TABLE II. Atomic Coordinates (10^4) with Their Standard Deviations in Parentheses and Equivalent Isotropic Temperature Factors

Atom	x	y	z	B_{eq}	Atom	x	y	z	B_{eq}
C(1)	9493 (3)	7770 (11)	11903 (11)	3.0	O(1)	9076 (4)	7279 (7)	10668 (7)	3.0
C(2)	10144 (6)	6587 (10)	12428 (10)	2.5	O(2)	9315 (5)	9007 (9)	12372 (9)	4.4
C(3)	10100 (6)	5391 (11)	11579 (10)	2.8	O(3)	10630 (5)	6876 (8)	13637 (7)	3.2
C(4)	9391 (6)	5764 (11)	10404 (10)	2.6	O(4)	8632 (6)	2561 (10)	12541 (9)	5.1
C(5)	8485 (6)	4727 (10)	10406 (10)	2.3	O(5)	8274 (4)	5011 (7)	7929 (6)	2.2
C(6)	7770 (6)	5114 (9)	9223 (9)	1.8	O(6)	7994 (6)	7474 (9)	7465 (9)	4.9
C(7)	6886 (6)	4065 (10)	9201 (10)	2.3	O(7)	7251 (4)	2509 (8)	9072 (7)	2.8
C(8)	6190 (6)	4309 (11)	7964 (11)	2.8	O(8)	6351 (6)	1813 (10)	10931 (9)	5.1
C(9)	5293 (7)	3309 (13)	8148 (13)	4.0	O(9)	5879 (4)	5907 (8)	8105 (7)	3.0
C(10)	11219 (8)	5655 (15)	14160 (12)	4.4	O(10)	5934 (7)	6212 (11)	5752 (9)	5.5
C(11)	8056 (7)	3612 (12)	12680 (11)	3.4	O(11)	4581 (4)	3770 (8)	7084 (8)	3.4
C(12)	7392 (9)	3748 (15)	13901 (12)	4.4	O(12)	3560 (5)	2046 (10)	7915 (9)	4.5
C(13)	8398 (6)	6313 (11)	7175 (11)	3.0	H(C3)	1052 (7)	437 (12)	1078 (10)	4.0
C(14)	9079 (8)	6104 (15)	5993 (11)	4.0	H(C4)	976 (6)	569 (12)	940 (10)	3.6
C(15)	6957 (7)	1540 (12)	10061 (10)	3.0	H(C5)	872 (7)	357 (12)	1027 (10)	3.9
C(16)	7494 (9)	60 (13)	9912 (14)	4.6	H(C6)	751 (6)	626 (13)	934 (10)	3.9
C(17)	5764 (7)	6659 (13)	6883 (11)	3.7	H(C7)	650 (7)	426 (12)	1016 (10)	3.7
C(18)	5467 (9)	8299 (14)	7204 (15)	5.2	H(C8)	656 (7)	400 (13)	698 (10)	3.8
C(19)	3719 (7)	3106 (13)	7146 (11)	3.5	H(C9)	547 (6)	213 (13)	801 (10)	3.9
C(20)	2995 (8)	3801 (16)	6159 (13)	4.5	H′(C9)	499 (7)	347 (12)	918 (10)	3.6
N	7986 (5)	4777 (8)	11733 (8)	2.4					

TABLE III. Bond Lengths (Å) with Their Standard Deviations in Parentheses

Bond	Length	Bond	Length
C(1)–C(2)	1.46 (1)	C(10)–O(3)	1.43 (1)
C(1)–O(1)	1.37 (1)	C(11)–C(12)	1.50 (2)
C(1)–O(2)	1.21 (1)	C(11)–N	1.37 (1)
C(2)–C(3)	1.33 (1)	C(11)–O(4)	1.23 (1)
C(2)–O(3)	1.35 (1)	C(13)–C(14)	1.49 (1)
C(3)–C(4)	1.51 (1)	C(13)–O(5)	1.37 (1)
C(4)–C(5)	1.54 (1)	C(13)–O(6)	1.20 (1)
C(4)–O(1)	1.43 (1)	C(15)–C(16)	1.51 (2)
C(5)–C(6)	1.52 (1)	C(15)–O(7)	1.34 (1)
C(5)–N	1.45 (1)	C(15)–O(8)	1.21 (1)
C(6)–C(7)	1.53 (1)	C(17)–C(18)	1.54 (2)
C(6)–O(5)	1.43 (1)	C(17)–O(9)	1.35 (1)
C(7)–C(8)	1.52 (1)	C(17)–O(10)	1.18 (1)
C(7)–O(7)	1.47 (1)	C(19)–C(20)	1.50 (2)
C(8)–C(9)	1.53 (1)	C(19)–O(11)	1.32 (1)
C(8)–O(9)	1.48 (1)	C(19)–O(12)	1.21 (1)
C(9)–O(11)	1.45 (1)		

MULTAN.[7] The E-map of the phase set with the highest figure of merit showed the skeleton of the molecule. The structure, thus obtained, was refined by a full-matrix least-squares method with anisotropic temperature factors. After all the hydrogen atoms had been located in the difference Fourier map, several cycles of least-squares refinement were carried out including these hydrogen atoms. The atomic scattering factors were those given by the International Tables for X-ray Crystallography.[8] The final R value was 8.0%, where $R=\Sigma||F_o|-|F_c||/\Sigma|F_c|$.

The final atomic parameters are listed in Table II. The absolute configurations of the

TABLE IV. Bond Angles (°) with Their Standard Deviations in Parentheses

C(2)–C(1)–O(1)	108.2 (8)	C(8)–C(9)–O(11)	106.8 (8)
C(2)–C(1)–O(2)	130.4 (9)	C(12)–C(11)–N	114.6 (9)
O(1)–C(1)–O(2)	121.4 (8)	C(12)–C(11)–O(4)	123.1 (10)
C(1)–C(2)–C(3)	109.8 (8)	N–C(11)–O(4)	122.2 (9)
C(1)–C(2)–O(3)	116.7 (8)	C(14)–C(13)–O(5)	112.3 (8)
C(3)–C(2)–O(3)	133.4 (8)	C(14)–C(13)–O(6)	125.4 (10)
C(2)–C(3)–C(4)	107.5 (8)	O(5)–C(13)–O(6)	122.3 (9)
C(3)–C(4)–C(5)	112.2 (7)	C(16)–C(15)–O(7)	109.5 (8)
C(3)–C(4)–O(1)	105.4 (7)	C(16)–C(15)–O(8)	125.5 (10)
C(5)–C(4)–O(1)	108.0 (6)	O(7)–C(15)–O(8)	125.1 (9)
C(5)–C(6)–C(7)	112.0 (7)	C(18)–C(17)–O(9)	108.6 (9)
C(4)–C(5)–N	112.1 (7)	C(18)–C(17)–O(10)	123.9 (11)
C(6)–C(5)–N	109.4 (6)	O(9)–C(17)–O(10)	127.3 (10)
C(5)–C(6)–C(7)	112.1 (6)	C(20)–C(19)–O(11)	112.0 (9)
C(5)–C(6)–O(5)	108.1 (6)	C(20)–C(19)–O(12)	125.2 (9)
C(7)–C(6)–O(5)	110.2 (6)	O(11)–C(19)–O(12)	122.8 (8)
C(6)–C(7)–C(8)	114.3 (7)	C(5)–N–C(11)	121.6 (7)
C(6)–C(7)–O(7)	107.3 (6)	C(1)–O(1)–C(4)	109.0 (6)
C(8)–C(7)–O(7)	106.2 (7)	C(2)–O(3)–C(10)	115.1 (7)
C(7)–C(8)–C(9)	108.9 (8)	C(6)–O(5)–C(13)	117.8 (6)
C(7)–C(8)–O(9)	104.1 (7)	C(7)–O(7)–C(15)	115.6 (6)
C(9)–C(8)–O(9)	107.9 (7)	C(8)–O(9)–C(17)	114.9 (7)

asymmetric centers agree with those of a related molecule, methyl β-glycosidic neuraminic acid.[9] Figure 1 shows a perspective drawing of **6**. Bond lengths and angles are shown in Tables III and IV. No abnormal lengths or angles were found in the structure.

The absolute configuration at C-4 was assigned as *S*. Torsional angles C(3)–C(4)–C(5)–C(6), C(4)–C(5)–C(6)–C(7), C(5)–C(6)–C(7)–C(8), and C(6)–C(7)–C(8)–C(9) are 180°, 170°, −175°, and −174°, respectively. The vicinal coupling constants between the hydrogen atoms on the side chain of **6**, shown in Table I, indicate that the side chain has the same zigzag conformation both in the crystal and in solution.

Experimental

General Methods——Melting points were determined in a capillary tube and are uncorrected. ^{1}H-NMR spectra were measured on a Varian XL-400 spectrometer with Me_4Si (TMS) as an internal standard in $CDCl_3$ or $Me_3Si(CH_2)_3SO_3Na$(DSS) in D_2O. The FD-MS were obtained on a JEOL JMS-DX 300 instrument, and IR spectra on a JASCO-A2 spectrometer. Optical rotations were measured with a JASCO-JIP-4 digital polarimeter. Column chromatography was performed on Wakogel C-200, and TLC was performed on Kieselgel 60 GF_{254} (Merck) with detection by ultraviolet (UV) light and by carrying with sulfuric acid.

Benzyl 5-Acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (2)——A solution of **1** (2 g, 6.47 mmol) in water (10 ml) was neutralized with 10% Cs_2CO_3 aqueous solution. The solution was lyophilized and dried over P_2O_5 to give the cesium salt of **1**, which was dissolved in dry DMF (20 ml). Then BnBr (5 ml) was added to the stirred solution, and the reaction mixture was stirred under argon for 2 h at room temperature. The reaction mixture was filtered and evaporated to a syrup, which was purified by recrystallization from 2-propanol to give 2.0 g (80%) of **2** as colorless needles. mp 183—185 °C, $[\alpha]_D^{28}$ −43° (c=1, H_2O). *Anal.* Calcd for $C_{17}H_{25}NO_9$: C, 52.71; H, 6.51; N, 3.28. Found: C, 52.68; H, 6.55; N, 3.24. IR ν_{max}^{KBr} cm^{-1}: 1710, 1620, 1520. ^{1}H-NMR (400 MHz, D_2O) δ_H (DSS): 1.822 (1H, dd, J=11.5, 13.0 Hz, 3-H_{ax}), 1.960 (3H, s, NAc), 2.233 (1H, dd, J=5.0, 13.0 Hz, 3-H_{eq}), 3.982 (1H, ddd, J=5.0, 10.5, 10.5 Hz, 4-H), 3.827 (1H, dd, J=10.2, 10.2 Hz, 5-H), 3.985 (1H, dd, J=10.5, 1.2 Hz, 6-H), 3.469 (1H, dd, J=1.2, 9.4 Hz, 7-H), 3.634 (1H, ddd, J=2.8, 6.1, 9.4 Hz, 8-H), 3.573 (1H, dd, J=6.1, 12.0 Hz, 9-H), 3.473 (1H, dd, J=2.8, 12.0 Hz, 9-H′), 5.219 (1H, d, J=12.0 Hz, phenyl-CH–), 5.230 (1H, J=12.0 Hz, phenyl-CH–), 7.038 (phenyl group).

5-Acetamido-2-*O*-benzyl-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (3)——1: Cs_2CO_3 (4.89 g, 15 mmol) and BnBr (2.38 g, 20 mmol) were added to a solution of **1** (1.55 g, 5 mmol) in DMF (20 ml) with stirring. After

2h at 40 °C, insoluble materials were removed through Celite, and the filtrate was evaporated. The residue was purified by recrystallization from ethanol to give 1.42 g (78%) of **3** as colorless crystals. mp 195—196 °C, $[\alpha]_D^{24}$ −64 ° (c=1, MeOH). MS (FD) m/z: 382 (M^++1). *Anal.* Calcd for $C_{18}H_{23}NO_8$: C, 56.29; H, 6.04; N, 3.67. Found: C, 56.83; H, 6.07; N, 3.64. IR ν_{max}^{KBr} cm^{-1}: 1765, 1650, 1535. ^{1}H-NMR data are shown on Table I.

2: A solution of **2** (0.11 g, 0.3 mmol) in DMF (10 ml) was treated with BnBr (0.5 ml). Work-up as described above afforded **3** (0.09 g, 80%). The physical constants were identical with those of **3** prepared above.

5-Acetamido-2-*O*-allyl-3,5-dideoxy-*β*-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (4)——Cs_2CO_3 (4.89 g, 15 mmol) and allyl bromide (2.19 ml, 20 mmol) were added to a solution of **1** (1.55 g, 5 mmol) in DMF (20 ml). The mixture was stirred for 3 h at 40 °C, insoluble materials were removed through Celite and the filtrate was evaporated. The residue was triturated with ethanol (100 ml), and the resulting insoluble materials were removed by filtration. After evaporation of the filtrate, the crude product was purified by silica gel column chromatography, with chloroform–methanol (4:1), and the product was recrystallized from ethanol to give 0.72 (42%) of **4** as colorless needles. mp 151—152 °C. $[\alpha]_D^{28}$ −77 ° (c=1, MeOH). MS (FD) m/z: 332 (M^++1). *Anal.* Calcd for $C_{14}H_{21}NO_8$: C, 50.76; H, 6.34; N, 4.23. Found: C, 50.28; H, 6.53; N, 4.18. IR ν_{max}^{KBr} cm^{-1}: 1775, 1640, 1540. ^{1}H-NMR data are given in Table I.

5-Acetamido-3,5-dideoxy-2-*O*-methyl-*β*-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (5)——Cs_2CO_3 (4.89 g, 15 mmol) and MeI (1.25 ml, 20 mmol) were added to a stirred solution of **1** (1.55 g, 5 mmol) in DMF. The mixture was stirred for 1.5 h at 40 °C, insoluble materials were removed through Celite and the filtrate was evaporated. The residue was triturated with ethanol (100 ml), and the resulting insoluble materials were removed by filtration. After evaporation of the filtrate, the crude product was purified by silica gel column chromatography with chloroform–methanol (4:1), and the product was recrystallized from ethanol to give 0.76 g (50%) of **5** as colorless prisms. mp 169.0—169.5 °C. $[\alpha]_D^{24}$ −79 ° (c=1, MeOH). MS (FD) m/z: 306 (M^++1). *Anal.* Calcd for $C_{12}H_{19}NO_8$: C, 47.21; H, 6.28; N, 4.59. Found: C, 47.06; H, 6.29; N, 4.59. IR ν_{max}^{KBr} cm^{-1}: 1770, 1650, 1535. ^{1}H-NMR data are given in Table I.

5-Acetamido-6,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-*O*-methyl-*β*-D-*glycero*-D-*galacto*-2-noneno-1,4-lactone (6)——Acetic anhydride (1 ml) was added to a solution of **5** (0.31 g, 1 mmol) in pyridine (1 ml). The reaction mixture was stirred overnight at room temperature, ethanol (10 ml) was added, and the whole was evaporate to dryness. The residue was purified by recrystallization from acetone to give 0.35 g (75%) of **6** as colorless prisms. mp 181—182 °C. $[\alpha]_D^{27}$ +13.8 ° (c=1, $CHCl_3$). *Anal.* Calcd for $C_{20}H_{27}NO_{12}$: C, 50.74; H, 5.71; N, 2.96. Found: C, 50.64; H, 5.75; N, 2.90. IR ν_{max}^{KBr} cm^{-1}: 1770 (sh), 1750, 1660, 1530. ^{1}H-NMR data are given in Table I.

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Homoisoflavonoids and Related Compounds. V.[1] A Novel Dibenzoxocin Derivative from *Caesalpinia sappan* L.

MICHIO NAMIKOSHI,* HIROYUKI NAKATA and TAMOTSU SAITOH

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

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A novel dibenzoxocin derivative, 7,8-dihydro-10-methoxy-3,7,11-trihydroxy-6*H*-dibenz[*b*,*d*]-oxocin-7-methanol was isolated from Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L. The chemical structure was established on the basis of the spectrometric data and the chemical interrelation with protosappanin B (7,8-dihydro-3,7,10,11-tetrahydroxy-6*H*-dibenz[*b*,*d*]oxocin-7-methanol).

Keywords——*Caesalpinia sappan*; Sappan Lignum; Leguminosae; heartwood; dibenzoxocin derivative; 10-*O*-methylprotosappanin B; ^{1}H–^{1}H difference NOE spectrum

Sappan Lignum, the dried heartwood of *Caesalpinia sappan* L., is a well known oriental crude drug, and has been used as an emmenagogue, hemostatic and antiinflammatory agent, as well as for the treatment of contusion and thrombosis.[2,3] Sappan Lignum and its main component, brazilin,[4c,5] have interesting pharmacological activities.[3]

We have investigated the chemical constituents of *C. sappan* in the course of our studies on homoisoflavonoids and related compounds, and reported the isolation and absolute stereochemistries of several phenolic constituents.[4] In a previous paper,[5] we clarified the chemotaxonomic relationships between *C. sappan* and *C. japonica* (the latter grows in Japan). Both species contain characteristic structural types of phenolic components, such as chalcones,[4b,5,6] flavonoids,[4b,7] homoisoflavonoids,[1,4,5] brazilins,[4c,5] dibenzoxocin derivatives[5,8] and so on.[9]

We have been further studying the isolation, absolute stereochemistries and biosyntheses of the phenolic constituents of *C. sappan* and *C. japonica*. In the present paper, we report the isolation and structural elucidation of a novel dibenzoxocin derivative, 10-*O*-methylprotosappanin B (**1**), from Sappan Lignum.

10-*O*-Methylprotosappanin B (**1**) was obtained by repeated silica gel and Sephadex LH-20 column chromatography, and preparative thin-layer chromatography (PTLC) of the remaining fractions after separation of the previously reported compounds.[4] The isolation of the intact compound was so difficult that only a limited amount could be obtained. However, a satisfactory amount was obtained in the form of an isopropylidene derivative (**6**) from the methanolic extract separated after treatment with acetone in the presence of an acid catalyst, as described in the previous paper.[4c] The remaining fractions after isolation of the isopropylidene derivatives of 3,4-dihydroxy homoisoflavans[4c] were subjected to column chromatography on silica gel and Sephadex LH-20 to yield **6**, together with the isopropylidene derivative of protosappanin B (**7**). On acid hydrolysis with 60% AcOH, compound **6** was readily transformed into **1**.

10-*O*-Methylprotosappanin B (**1**), $[\alpha]_D$ $-11.2°$ (MeOH) had the molecular formula $C_{17}H_{18}O_6$ by high-resolution mass spectrometry (MS) and showed broadening of the signals in the 100 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectrum observed at room

1: R_1=Me, R_2=R_3=H
2: R_1=Me, R_2=R_3=Ac
3: R_1=R_2=R_3=H
4: R_1=R_2=R_3=Ac
5: R_1=R_2=Me, R_3=H

6: R_1=Me, R_2=H
7: R_1=R_2=H
8: R_1=R_2=Me

Chart 1

temperature. In the ultraviolet (UV) spectrum of **1**, two absorption maxima at 286 and 254 nm appeared, as in the case of protosappanin B (**3**),[5,8b] suggesting that **1** has a 7,8-dihydro-6*H*-dibenzoxocin skeleton. The compound appears to exist in two conformers of the 8-membered ring (C-ring), since the 400 MHz ^{1}H-NMR spectrum of **1** revealed the presence of two pairs of signals. Compound **6**, $[\alpha]_D$ $-10.0°$ (MeOH), had the molecular formula $C_{20}H_{22}O_6$ (high-resolution MS) and showed broadening of the signals in the 100 MHz ^{1}H-NMR spectrum as in the case of **1**. While the signals were not observed separately, the ^{1}H-NMR spectra of **1** and **6** resembled those of **3** and **7**, respectively, except for phenolic methoxy methyl signals in **1** (δ 3.78) and **6** (δ 3.88).

Compound **1** afforded a triacetyl derivative (**2**) on acetylation with Ac_2O–NaOAc. The 400 MHz, ^{1}H-NMR spectrum of **2** revealed that the conformation of the C-ring was predominantly in one form (about 85%; discussed later) as shown in Fig. 1(A). A similar phenomenon was observed with **4**, the tetraacetyl derivative of **3**. The ^{1}H-NMR spectrum of **2** (data are summarized in Table I) indicated the presence of three isolated methylene groups with signals at δ 3.90 and 4.34 (each 1H, d, J=12.0 Hz), 4.17 (2H, s), and 2.71 and 2.78 (each 1H, d, J=14.0 Hz), and a phenolic methoxy methyl group (three-proton singlet at δ 3.88). In addition, it showed two one-proton singlets at δ 6.90 and 7.04, which indicated the presence of a 1,2,4,5-tetrasubstituted benzene ring. Characteristic ABX-type aromatic proton signals were observed at δ 6.85 (1H, d, J=2.3 Hz), 6.89 (1H, dd, J=8.0, 2.3 Hz) and 7.26 (1H, d, J= 8.0 Hz), assignable to the protons at C-4, C-2 and C-1, respectively.

On methylation with dimethyl sulfate (Me_2SO_4), **6** afforded a trimethyl ether (**8**), and **7** gave the same compound (**8**). Moreover, **8** derived from **6** was hydrolyzed to form **5** upon heating in 80% AcOH. The chromatographic and physicochemical properties of this compound coincided with those of **5** derived from **3** upon methylation with diazomethane.

On the basis of the above facts, compound **1** was deduced to be the monomethyl derivative of protosappanin B (**3**). The ^{1}H-NMR data for **2** closely resembled those for **4** except for a methoxy methyl singlet at δ 3.88 in the spectrum of **2** (Table I). ^{1}H-NMR signals due to the protons at C-1, C-2 and C-4 of **2** were observed at almost the same magnetic fields as those of **4**. However, the signals due to H-9 and H-12 of **2** were shifted upfield as compared with those of **4**. The methoxyl group of **2** was therefore indicated to be attached to either C-10 or C-11 on the B-ring.

In order to determine the exact position of the methoxyl group, ^{1}H–^{1}H difference nuclear Overhauser effect (NOE) spectra (Fig. 1(B) and (C)) were measured with a 400 MHz ^{1}H-NMR spectrometer. The proton signal due to H-9 was identified first by irradiation of the C-8 methylene proton (δ 2.74). An NOE enhancement was detected at δ 6.90 (Fig. 1(B)), which was therefore assigned to the H-9 proton. Subsequently, the methoxy methyl group (δ 3.88) was irradiated and an NOE enhancement was again observed at δ 6.90 (Fig. 1(C)).

TABLE I. ¹H-NMR Data for Compounds **2** and **4** in $CDCl_3$[a]

	2	**4**
H-1	7.26, 1H, d, J=8.0	7.28, 1H, d, J=8.0
H-2	6.89, 1H, dd, J=8.0, 2.3	6.90, 1H, dd, J=8.0, 2.3
H-4	6.85, 1H, d, J=2.3	6.86, 1H, d, J=2.3
H-6	3.90, 1H, d, J=12.0	3.89, 1H, d, J=12.0
	4.34, 1H, d, J=12.0	4.30, 1H, d, J=12.0
7-CH_2-	4.17, 2H, s	4.13, 2H, s
H-8	2.71, 1H, d, J=14.0	2.67, 1H, d, J=14.0
	2.78, 1H, d, J=14.0	2.80, 1H, d, J=14.0
H-9	6.90, 1H, s	7.15, 1H, s
H-12	7.04, 1H, s	7.18, 1H, s
10-OMe	3.88, 3H, s	—

a) Chemical shifts are given in δ relative to tetramethylsilane. Coupling constants are given in Hz. Acetyl methyl signals are excluded.

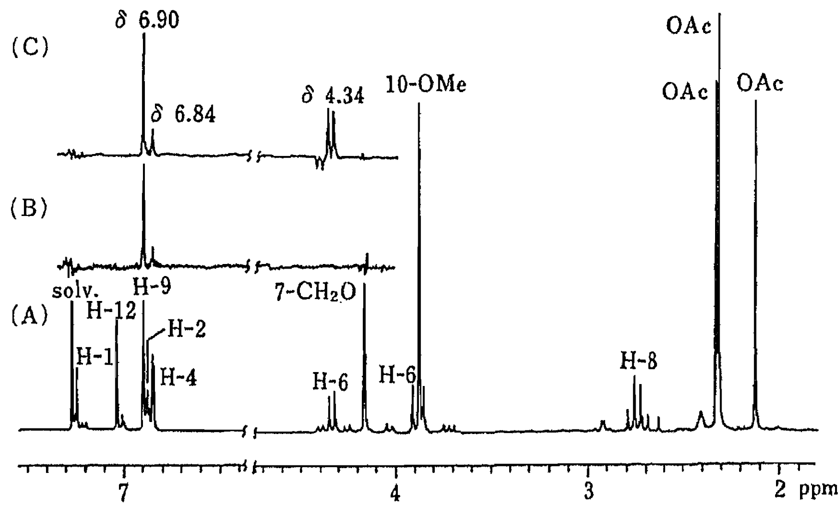

Fig. 1. The ¹H-NMR Spectrum (A) and ¹H-¹H Difference NOE Spectra (B and C) of Compound **2** (400 MHz)

(A) Non-decoupled. (B) Irradiated at δ2.74 (H-8). (C) Irradiated at δ3.88 (10-OMe).

Consequently, the methoxyl group should be attached to the C-10 position on the B-ring of **2**.

10-*O*-Methylprotosappanin B (**1**) is therefore 7,8-dihydro-10-methoxy-3,7,11-trihydroxy-6*H*-dibenz[*b*,*d*]oxocin-7-methanol, as shown in Chart 1.

A singlet at δ 6.84 in the difference NOE spectra of **2** (Fig. 1(B) and (C)) is ascribable to the H-9 proton in the other conformer of the C-ring. Similarly, the signal of the proton at C-12 was observed as a pair of signals at δ 7.04 and 7.01 (about 6 : 1). The remaining signals were also accompanied with corresponding signals originating from the other conformer, and the integrations were consistent with a ratio of the two conformers of about 6 : 1. An NOE enhancement was detected at the doublet at δ 4.34 as well as the H-9 signal at δ 6.90 on irradiation at δ 3.88 (10-OMe), as shown in Fig. 1(C), owing to the irradiation of one of the methylene protons at C-6 (δ 3.90) simultaneously with the 10-OMe (δ 3.88).

Dibenzoxocin derivatives were proposed by Nagai *et al.* to be biosynthesized from homoisoflavonoids.[8c] From *C. japonica*, we obtained two biphenyl compounds[5] whose structures have not yet been determined exactly, but these compounds are considered to be candidates for the biogenetic precursors of dibenzoxocin derivatives. *C. japonica* contains a

larger quantity of dibenzoxocin derivatives in comparison with brazilin and homoisoflavonoids, in contrast with *C. sappan*.[5] Thus, we are studying the biosynthesis of dibenzoxocin compounds in the fresh wood of *C. japonica*, and will report elsewhere on the structures of the biphenyl compounds mentioned above. The absolute stereochemistries of dibenzoxocin derivatives are also under investigation in our laboratory.

Experimental

General——See reference 4*c*.

Extraction and Isolation——Sappan Lignum, the dried heartwood of *C. sappan* L., purchased in Tokyo, was extracted with MeOH and the extract was separated as described in the previous papers.[4] Compound **1** was obtained by repeated column chromatography on silica gel and Sephadex LH-20 (MeOH), and PTLC of the remaining fractions after separation of the previously reported compounds.[4]

The methanolic extract (52.6 g) of Sappan Lignum was treated with acetone at room temperature in the presence of Amberlyst 15 as reported previously.[4c] Compounds **6** (132 mg) and **7** were isolated from the remaining fractions described in ref. 4*c* by column chromatography on silica gel and Sephadex LH-20.

10-*O*-Methylprotosappanin B (1)——A white powder. $[\alpha]_D^{25}$ $-11.2°$ ($c=1.82$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 286 (4.26), 254 (4.40). EI-MS *m/z*: 318.1071 (M^+, Calcd for $C_{17}H_{18}O_6$: 318.1101), 257, 246, 244, 243, 229, 227, 213, 201. ^{1}H-NMR (CD_3OD) δ: 2.55—2.84 (2H, H-8), 3.35—4.36 (4H, H-6 and 7-CH_2O), 3.78 (3H, s, 10-OMe), 6.54 (1H, br s, H-4), 6.59 (1H, dd, $J=8.0$, 2.0 Hz, H-2), 6.77 (1H, br s, H-9), 6.89 (1H, br s, H-12), 7.03 (1H, d, $J=8.0$ Hz, H-1).

From Compound **6**: A solution of **6** (58 mg) in 60% AcOH was heated at 60 °C for 3 h. The solvents were evaporated off and the residue was purified by PTLC ($CHCl_3$–MeOH = 9 : 1) to afford **1** (52 mg).

Compound 2——10-*O*-Methylprotosappanin B (**1**) (25 mg) was acetylated with Ac_2O–NaOAc at 80 °C for 20 min, and the product was purified by PTLC (benzene–acetone = 9 : 1) to yield **2** (29 mg). $[\alpha]_D^{25}$ $-57.7°$ ($c=0.31$, $CHCl_3$). EI-MS *m/z*: 444 (M^+), 402, 360, 244, 243.

Protosappanin B (3)——From Compound **7**: Compound **7** (36 mg) was treated as described for **1** to afford **3** (32 mg). The physicochemical properties of **3** coincided with those of the natural product.[5]

Compound 4——Compound **3** (62 mg) was acetylated as described for **2** to yield **4** (82 mg), $[\alpha]_D^{25}$ $-36.5°$ ($c=2.86$, $CHCl_3$). EI-MS *m/z*: 472 (M^+), 430, 388, 346, 304.

Compound 5——From Protosappanin B (**3**): A solution of **3** (570 mg) in MeOH was treated with CH_2N_2/Et_2O and the mixture was allowed to stand at 4 °C for 22 h. After removal of the solvents, the residue was subjected to silica gel column chromatography with benzene–acetone (4 : 1) to afford **5** (132 mg), $[\alpha]_D^{25}$ $-52.4°$ ($c=2.13$, $CHCl_3$). EI-MS *m/z*: 346 (M^+), 272, 271, 257. ^{1}H-NMR ($CDCl_3$) δ: 2.58—3.00 (2H, H-8), 3.59 (2H, br s, 7-CH_2O), 3.83, 3.90, 3.92 (each 3H, s, OMe × 3), 4.35 (1H, br d, $J=11.5$ Hz, H-6), 6.65 (1H, d, $J=2.5$ Hz, H-4), 6.74 (1H, dd, $J=9.0$, 2.5 Hz, H-2), 6.82, 6.87 (each 1H, s, H-9 and 12), 7.22 (1H, d, $J=9.0$ Hz, H-1). The dimethyl derivative, the tetramethyl derivative and an unknown compound were obtained as by-products.

From Compound **8**: A solution of **8** (221 mg) in 80% AcOH was treated as described for **1** to give **5** (182 mg), $[\alpha]_D^{25}$ $-50.2°$ ($c=2.01$, $CHCl_3$). The physicochemical properties of this compound coincided with those of **5** derived from **3**.

Compound 6——A white powder, $[\alpha]_D^{25}$ $-10.0°$ ($c=2.20$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 286 (3.92), 255 (4.11). EI-MS *m/z*: 358.1413 (M^+, Calcd for $C_{20}H_{22}O_6$: 358.1414), 275, 273, 257, 246, 243, 242, 229. ^{1}H-NMR (acetone-d_6) δ: 1.26—1.60 (6H, $C(CH_3)_2$), 2.68—3.00 (2H, H-8), 3.42—4.40 (4H, H-6 and 7-CH_2O), 3.88 (3H, s, 10-OMe), 6.35—6.90 (4H, H-2, 4, 9 and 12), 7.01 (1H, d, $J=9.0$ Hz, H-1).

From 10-*O*-Methylprotosappanin B (**1**): A solution of **1** (12 mg) in dry acetone (10 ml) was stirred at room temperature for 16 h in the presence of Amberlyst 15. After filtration and evaporation, the product was purified by PTLC ($CHCl_3$–MeOH = 9 : 1) to afford **6** (13 mg).

Compound 7——A white powder, $[\alpha]_D^{25}$ $-9.3°$ ($c=2.04$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 288 (4.00), 255 (4.16). EI-MS *m/z*: 344.1260 (M^+, Calcd for $C_{19}H_{20}O_6$: 344.1260), 286, 269, 244, 243, 232, 230, 229, 227. ^{1}H-NMR (CD_3OD) δ: 1.30—1.56 (6H, $C(CH_3)_2$), 2.60—2.90 (2H, H-8), 3.58—4.30 (4H, H-6 and 7-CH_2O), 6.35—6.75 (4H, H-2, 4, 9 and 12), 7.00 (1H, br d, $J=9.0$ Hz, H-1).

From Protosappanin B (**3**): Compound **3** (34 mg) was treated as described for **6** to yield **7** (32 mg).

Compound 8——From Compound **7**: A mixture of **7** (586 mg), Me_2SO_4 (780 mg), anhydrous K_2CO_3 (2 g) and dry acetone (30 ml) was refluxed for 3 h, and then filtered and evaporated. The residue was washed sufficiently with H_2O and dried. The product was purified by silica gel column chromatography with benzene–acetone (97 : 3) to yield **8** (638 mg), $[\alpha]_D^{25}$ $-37.6°$ ($c=4.82$, $CHCl_3$). EI-MS *m/z*: 386 (M^+), 328, 313, 274, 272, 271, 257. ^{1}H-NMR ($CDCl_3$) δ: 1.30—1.70 (6H, $C(CH_3)_2$), 2.60—3.20 (2H, H-8), 3.60—4.50 (13H, H-6, 7-CH_2O and OMe × 3), 6.50—6.90 (4H, H-2, 4, 9 and 12), 7.16 (1H, d, $J=9.0$ Hz, H-1).

From Compound **6**: Compound **6** (32 mg) was methylated under similar conditions to give **8** (32 mg), $[\alpha]_D^{25}$ $-29.5°$ ($c=1.01$, $CHCl_3$). The physicochemical properties of the product coincided with those of **8** derived from **7**.

From Compound **5**: A solution of **5** (26 mg) in dry acetone was treated as described for **6**. The physicochemical properties of the product coincided with those of **8**.

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Reaction of Trifluoromethyl Ketones. IV.[1] Cyclization of the Ene Reaction Products of Trifluoromethyl Ketones: A Facile Synthesis of 2-(Trifluoromethyl)tetrahydrofuran[2]

TAKABUMI NAGAI, TAKUICHI MIKI, and ITSUMARO KUMADAKI*

Faculty of Pharmaceutical Sciences, Setsunan University, Nagaotoge-cho, Hirakata, Osaka 573–01, Japan

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1-(Trifluoromethyl)homoallyl alcohols, which were obtained by the ene reaction of trifluoromethyl ketones with olefins, were cyclized to 2-(trifluoromethyl)tetrahydrofurans in the presence of *p*-toluenesulfonic acid, a typical Brensted acid. The ease of this cyclization depends on the substituents on the homoallyl system. The stereochemistry of the cyclization products was established by analysis of the two dimensional nuclear Overhauser effects. The stable conformations of the tetrahydrofuran compounds were estimated by means of molecular mechanics calculations of the steric energy. The observed ratio of the products was consistent with that estimated from the steric energy calculations.

Keywords——trifluoromethyl; tetrahydrofuran; *p*-toluenesulfonic acid; homoallyl alcohol; cyclization; ene-reaction product; steric energy; molecular mechanics

The ene reaction of trifluoromethyl ketones has been investigated, and this reaction was found to be very useful for the synthesis of trifluoromethylated homoallyl alcohols.[1,3,4] We are now engaged in the derivatization of these homoallyl alcohols to various trifluoromethylated compounds. This paper is concerned with the cyclization of the homoallyl alcohols to 2-(trifluoromethyl)tetrahydrofurans. As mentioned in the previous papers,[1,3,4] these tetrahydrofurans were formed in only small amounts in the ene reaction, especially when aluminum chloride was used as a catalyst (see Chart 1).

Since 2-(trifluoromethyl)tetrahydrofurans are intrinsically interesting and also are useful as protected forms of trifluoromethylated 1,4-diols or homoallyl alcohols, we attempted the quantitative conversion of the ene adducts to the tetrahydrofuran compounds, and succeeded in this transformation.

Formation of the tetrahydrofurans during the ene reaction seemed to be caused by acid catalysis, since the tetrahydrofuran compounds were formed as by-products in the ene reactions in the presence of aluminum chloride. Therefore, the cyclization was tried in the

$CF_3-C(=O)-CF_3$ + allyl phenyl sulfide —Δ→ homoallyl alcohol (CF_3, OH, CF_3) + tetrahydrofuran (CF_3, CF_3)

$CF_3-C(=O)-CH_3$ + allylbenzene —$AlCl_3$→ homoallyl alcohol (CH_3, OH, CF_3) (major) + tetrahydrofuran (CF_3, CH_3) (minor)

Chart 1

presence of aluminum chloride. Treatment of the homoallyl alcohol with aluminum chloride at $-78\,^{\circ}C$ gave a mixture of the homoallyl alcohol and the tetrahydrofuran compound in the same ratio as that of the ene reaction catalyzed by aluminum chloride. Even when the reaction time was prolonged, the ratio remained unchanged, though both compounds decomposed and marked tar formation occurred. These results showed that aluminum chloride catalyzed the cyclization, but the reaction did not go to completion (see Chart 2). This may be due to the high affinity of aluminum chloride for alcohols.

$AlCl_3$, $-78\,^{\circ}C$, 1h → 4 : 1

$AlCl_3$, $-78\,^{\circ}C$, 1h → 3 : 1

1 → $BF_3 \cdot Et_2O$ in C_6H_6, reflux → **1** + **2** (1 : 1)

1 → HCl in MeOH, reflux → **1** + **2** (1 : 1)

1 → TsOH in C_6H_6, reflux → **2** 79%

Chart 2

As 1,1,1-trifluoro-5-phenylthio-2-(trifluoromethyl)-4-penten-2-ol (**1**) was cyclized to the tetrahydrofuran compound simply by heating it at high temperature, the reaction of this compound was examined at a lower temperature in the presence of various acids to find a good catalyst. Treatment of **1** with boron trifluoride etherate resulted in the formation of an equilibrium mixture of **1** and the tetrahydrofuran compound (**2**) in a ratio of approximately 1 : 1.

Since Lewis acids seemed to form stable complexes with homoallyl alcohols, reactions in the presence of protic acids were examined. The reaction of **1** in the presence of hydrochloric acid in methanol still gave an equilibrium mixture of **1** and **2** in a ratio of 1 : 1. As the formation of a hydrogen bond of **1** with the hydroxyl group of methanol might favor the hydroxylic compound (**1**), *p*-toluenesulfonic acid was examined as a catalyst in an aprotic solvent, benzene. In this case, all the starting material was converted to the tetrahydrofuran compound in a high yield. This condition was found to be applicable to many kinds of trifluoromethylated homoallyl alcohols, as shown in Table I.

The reactions of **5** and **24** show that the homoallyl alcohols substituted with a phenyl group at the 4-position reacted faster than those without a phenyl group at this position. The initial rate of reaction of **10** was much faster than that of **12**. These results suggest that the cyclization occurs through a carbocation intermediate, which is stabilized by a phenyl group, as shown in Chart 3.

Namely, the 3-position of the homoallyl alcohol is protonated, a stable benzyl cation is formed, and this cation cyclizes to a tetrahydrofuran compound. This mechanism is supported by the fact that the cyclization of **3** was accelerated remarkably by the introduction

TABLE I. Cyclization of Homoallyl Alcohols with p-TsOH in Benzene

Starting material R^1	R^2	R^3	R^4	Refluxing time	Product[a)]	Yield (%)
p-$CH_3OC_6H_4$	CF_3	H	H (3)	1.5 h	4	59
C_6H_5	CF_3	H	H (5)	8 h	6	76
n-C_5H_{11}	CF_3	H	H (7)	4 d	8	48
H	CF_3	H	C_6H_5 (9)	1 d	(tar)	
C_6H_5	CH_3	H	H (10)	15 h	11	50 (3/2)[b)]
n-C_5H_{11}	CH_3	H	H (12)	15 h	13	70 (2/1)[b)]
n-C_7H_{15}	CH_3	H	H (14)	35 h	15	89 (2/1)[b)]
n-C_4H_9	CH_3	CH_3	H (16)	34 h	17	41 (1/3)[b)]
H	CH_3	H	C_6H_5 (18)	15 h	(19)	77
n-C_3H_7	n-C_4H_9	H	H (20)	50 h	21	80 (3/1)[b)]
n-C_5H_{11}	n-C_4H_9	H	H (22)	41 h	23	73 (5/2)[b)]
C_6H_5	n-C_4H_9	H	H (24)	15 h	25	88 (3/2)[b)]
n-C_3H_7	C_6H_5	H	H (26)	35 h	27	75 (3/2)[b)]
n-C_5H_{11}	C_6H_5	H	H (28)	30 h	29	89 (3/2)[b)]

a) All the products except 19 are tetrahydrofuran derivatives. *b)* Total yields of diastereomers are presented. The ratios of these isomers (*cis*/*trans* for R^1 and CF_3) are shown in parentheses. The two methyl groups of 17 are *cis* to each other.

R'=aliphatic

Chart 3

of a methoxy group on the benzene ring. Further, the homoallyl compound (**18**), which was substituted with a phenyl group at the 3-position, did not give a tetrahydrofuran compound, but gave a dehydrated product. In this case, a benzyl cation would have been formed, but the hydroxylic oxygen was not at a suitable position for intramolecular cyclization, so that the dehydration occurred. The facile cyclization of **1** may be explained as follows; a phenylthio group stabilizes the cation intermediate due to the lone pair electrons on the sulfur atom and facilitates the cyclization.

The structures of the tetrahydrofuran compounds were determined by analysis of the two-dimensional nuclear Overhauser effects (NOE). For example, the NOE spectrum of one

product (**11a**) from **10** showed an association between the methyl and the phenyl protons, while that of the other product (**11b**) did not. This finding shows that the methyl and the phenyl groups of **11a** are *cis* to each other. This conclusion agrees with the earlier estimation of the structures of **11a** and **11b** based on the chemical shifts in proton magnetic resonance (^{1}H-NMR) spectroscopy.[3]

The tetrahydrofurans substituted with a phenyl group at the 5-position, except **4** and **6**, were obtained as a mixture of diastereoisomers either from the aluminum chloride-catalyzed ene reaction or from the *p*-toluenesulfonic acid-catalyzed cyclization. On the other hand, while one diastereoisomer was obtained in the ene reaction of olefinic compounds without a phenyl group catalyzed by aluminum chloride at −78 °C, two diastereoisomers were obtained in the *p*-toluenesulfonic acid-catalyzed cyclization. Thus, while the ene reaction of 1-octene with trifluoroacetone catalyzed by aluminum chloride gave **12** with one tetrahydrofuran (**13a**),[3] the cyclization of **12** in the presence of *p*-toluenesulfonic acid gave two isomers (**13a** and **13b**, ratio 2 : 1). The NOE analysis of **13a** showed a strong association between the 5-H and the 2-methyl protons. Namely, these groups are *cis* to each other. Since a trifluoromethyl group is sterically larger than a methyl group, the preferred formation of **13a**, where the trifluoromethyl group and the pentyl group are *cis* to each other, seems to be peculiar. However, estimation of the stabilities of **13a** and **13b** by molecular mechanics calculations showed that both compounds have approximately equal steric energies (**13a**, 17.20 kcal/mol; **13b**, 17.18 kcal/mol). In the most stable conformation of **13a** and **13b** estimated by MM2,[5] the oxygen atom and the 2-carbon atom of the tetrahydrofuran ring occupy the bottom of the envelope form, as shown in Fig. 1. The methyl and the trifluoromethyl groups are eclipsed to the lone pairs of the oxygen atom. A lone pair of electrons is considered to be the smallest substituent. Thus, the difference of configuration did not have much effect on the steric energy.

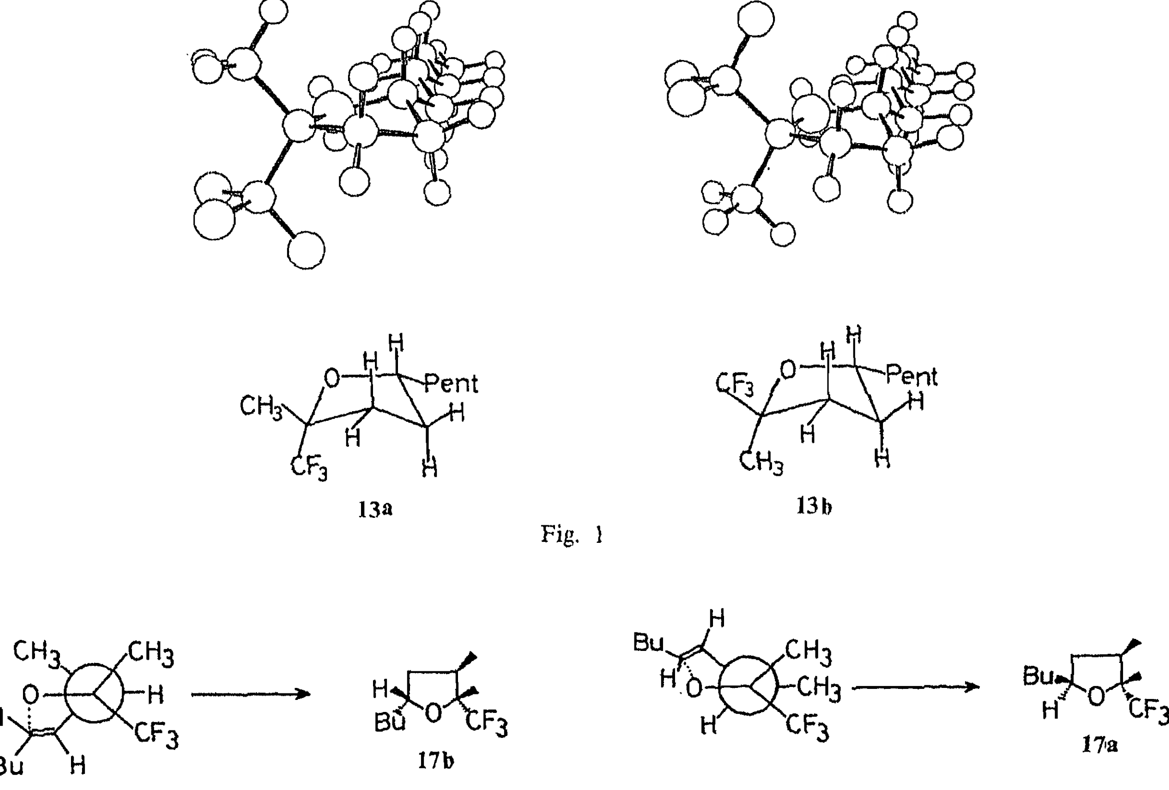

Fig. 1

Fig. 2

17a

17b

Fig. 3

The two-dimensional NOE of **17a** and **17b** showed a strong association between the protons of the 2-methyl group and those of the 3-methyl group. This fact suggests that the methyl groups are *cis* to each other and that the stereochemistry of the starting material (**16**) was 2*R**,3*R**. The minor product (**17b**) showed a strong NOE association between the proton at the 5-position and those of the 2-methyl group. This result confirmed that the stereochemistry of **17b** was 2*R**,3*R**,5*R**. The major product (**17a**) did not show this association and should be the 2*R**,3*R**,5*S**-isomer. The aluminum chloride-catalyzed ene reaction of *trans*-2-octene and trifluoroacetone gave **16** and **17b**, but not **17a**. The conformation of the intermediate to **17b** seems to be more stable than that to **17a**, since the methyl group at the 3-position is *gauche* to both the methyl and the trifluoromethyl group at the 2-position of the transition state to **17a**, as shown in Fig. 2. The kinetically controlled cyclization of this conformer at a low temperature gave **17b**. The estimation of the stabilities of **17a** and **17b** by molecular mechanics calculations showed that the former is more stable than the latter (18.17 kcal/mol for **17a**, and 19.74 kcal/mol for **17b**). The preferred formation of the stable isomer in the *p*-toluenesulfonic acid-catalyzed cyclization suggests that this reaction is a thermodynamically controlled equilibrium reaction. The nonstereoselectivity of this cyclization is in accordance with the mechanism involving a cationic intermediate as postulated above.

The stable conformations of **17a** and **17b** were estimated by molecular mechanics calculations as shown in Fig. 3. In the stable conformation of **17b**, carbon-2 occupies the tip of the flap of the envelope form of the tetrahydrofuran ring and the large trifluoromethyl group takes the quasi-equatorial position, while carbon-4 occupies the tip of the flap of the envelope and the butyl and the 3-methyl groups take quasi-equatorial positions in the stable conformer of **17a**. The steric energy of the latter was calculated to be smaller than that of the former by 1.5 kcal/mol, probably because the latter has no substituent at the quasi-axial position and no eclipsed substituents. In the former, the 2-methyl group is in a quasi-axial position and the relation between the 4- and 5-carbons is fully eclipsed.

In conclusion, 2-(trifluoromethyl)homoallyl alcohols were converted to 2-(trifluoromethyl)tetrahydrofurans of *p*-toluenesulfonic acid-catalyzed cyclization. This reaction gave a mixture of diastereoisomers, the major one of which was that expected from MM2 calculation. This fact suggests that this reaction is an equilibrium reaction through a

carbocation intermediate.

Experimental

Trifluoromethyl homoallyl alcohols were synthesized according to the literature.[1] ^{1}H-NMR spectra were obtained on JNM-FX90Q and JNM-GX400 spectrometers. ^{19}F-NMR spectra were recorded on JNM-FX90Q spectrometer, using benzotrifluoride as an internal standard (upper field taken as plus).

5-Phenylthio-2,2-bis(trifluoromethyl)tetrahydrofuran (2)——A mixture of 1,1,1-trifluoro-5-phenylthio-2-(trifluoromethyl)-4-penten-2-ol (**1**, 166 mg, 0.5 mmol) and *p*-TsOH·H_2O (100 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 1 h. The mixture was poured into saturated $NaHCO_3$ and extracted with ether. The ether layer was washed with water and dried over $MgSO_4$. After evaporation of the solvent under vacuum, the residue was purified through an SiO_2 column in CH_2Cl_2–hexane (1 : 10) solution to give **2** (131 mg, 79%). **2**: colorless oil, bp 95—110 °C (4 mmHg, bulb-to-bulb distillation). Msss spectrum (MS) *m/z*: 316 (M^+). High resolution mass spectrum (HRMS) Calcd for $C_{12}H_{10}F_6OS$; 316.035. Found: 316.036. ^{1}H-NMR ($CDCl_3$) δ: 1.83—2.77 (4H, m), 5.45—5.85 (1H, m), 7.06—7.80 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 13.90 (q, J=8.5 Hz), 14.42 (q, J=8.5 Hz).

5-(4-Methoxyphenyl)-2,2-bis(trifluoromethyl)tetrahydrofuran (4)——A mixture of 1,1,1-trifluoro-5-(4-methoxyphenyl)-2-(trifluoromethyl)-4-penten-2-ol (**3**, 155 mg, 0.5 mmol) and *p*-TsOH·H_2O (100 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 1.5 h. The reaction mixture was worked up in the same manner as in the case of **2**, and the residue was purified by column chromatography (SiO_2, CH_2Cl_2–hexane, 1 : 10) to give **4** (91.5 mg, 59%). **4**: colorless oil. MS *m/z*: 314 (M^+). HRMS Calcd for $C_{13}H_{12}F_6O_2$; 314.074. Found: 314.073. ^{1}H-NMR ($CDCl_3$) δ: 1.60—2.82 (4H, m), 4.01 (3H, s), 4.83—5.33 (1H, m), 6.83 (2H, d, J=8.7 Hz), 7.21 (2H, d, J=8.7 Hz). ^{19}F-NMR ($CDCl_3$) ppm: 14.14 (q, J=7.8 Hz), 14.54 (q, J=7.8 Hz).

5-Phenyl-2,2-bis(trifluoromethyl)tetrahydrofuran (6)——A mixture of 1,1,1-trifluoro-5-phenyl-2-(trifluoromethyl)-4-penten-2-ol (**5**, 142 mg, 0.5 mmol) and *p*-TsOH·H_2O (95 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 8 h. After a usual work-up of the reaction mixture, the residue was purified by column chromatography (SiO_2, hexane) to give **6** (107 mg, 76%). **6**: colorless oil. MS *m/z*: 284 (M^+). HRMS Calcd for $C_{12}H_{10}F_6O$: 284.064. Found: 284.063. ^{1}H-NMR ($CDCl_3$) δ: 1.87—3.10 (4H, m), 5.23—5.64 (1H, m), 7.30—7.66 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 14.10 (q, J=7.6 Hz), 14.47 (q, J=7.6 Hz).

5-Pentyl-2,2-bis(trifluoromethyl)tetrahydrofuran (8)——A mixture of 1,1,1-trifluoro-2-(trifluoromethyl)-4-decen-2-ol (**7**, 216 mg, 0.8 mmol) and *p*-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 4 d. After the usual work-up of the reaction mixture, the residue was purified by column chromatography (SiO_2, CH_2Cl_2–hexane, 1 : 10) to give **8** (104 mg, 48%). **8**: colorless oil. MS *m/z*: 278 (M^+). HRMS Calcd for $C_{11}H_{16}F_6O$: 278.111. Found: 278.111. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.1 Hz), 1.07—2.47 (12H, m), 4.02—4.42 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 14.33 (q, J=8.8 Hz), 14.94 (q, J=8.8 Hz).

An Attempted Cyclization of 1,1,1-Trifluoro-4-phenyl-2-(trifluoromethyl)-4-penten-2-ol (9)——A mixture of **9** (288 mg, 1.0 mmol) and *p*-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 21 h. Analysis of the reaction mixture by gas-liquid chromatography (GLC) showed a peak of starting material with many small peaks of unidentified products.

(2*R,5*R**)- (11a) and (2*R**,5*S**)-2-Methyl-5-phenyl-2-(trifluoromethyl)tetrahydrofuran (11b)**——A mixture of 1,1,1-trifluoro-2-methyl-5-phenyl-4-penten-2-ol (**10**, 238 mg, 1.0 mmol) and *p*-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 15 h. After the usual work-up of the reaction mixture, the residue was separated by column chromatography (SiO_2, CH_2Cl_2–hexane, 1 : 5) to give 2-methyl-5-phenyl-2-(trifluoromethyl)tetrahydrofuran (120 mg, 50%); colorless oil, bp 120 °C (17 mmHg, bulb-to-bulb distillation). MS *m/z*: 230 (M^+). HRMS Calcd for $C_{12}H_{13}F_3O$: 230.092. Found: 230.091. This was found to be a mixture of two diastereoisomers (ratio 2 : 3) from ^{19}F-NMR and separated into **11a** and **11b** by medium-pressure column chromatography (SiO_2, hexane–2-propanol, 1000 : 1). **11a**: ^{1}H-NMR ($CDCl_3$) δ: 1.51 (3H, d, J=0.9 Hz), 1.86—1.99 (2H, m), 2.35—2.43 (2H, m), 5.03—5.09 (1H, m), 7.23—7.35 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.30 (s). **11b**: ^{1}H-NMR ($CDCl_3$) δ: 1.49 (3H, q, J=1.0 Hz), 1.89—2.09 (2H, m), 2.22—2.34 (1H, m), 2.39—2.51 (1H, m), 5.00—5.05 (1H, m), 7.20—7.40 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.45 (s). The homonuclear two-dimensional Overhauser effect of **11a** showed an interaction between the 2-methyl and the 5-phenyl groups. This result showed that the two substituents are *cis*.

(2*R,5*R**)- (13a) and (2*R**,5*S**)-2-Methyl-5-pentyl-2-(trifluoromethyl)tetrahydrofuran (13b)**——A mixture of 1,1,1-trifluoro-2-methyl-4-decen-2-ol (**12**, 229 mg, 1.0 mmol) and *p*-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 15 h. After the usual work-up of the reaction mixture, distillation of the residue gave 2-methyl-5-pentyl-2-(trifluoromethyl)tetrahydrofuran (159 mg, 70%); a colorless oil, bp 80 °C (25 mmHg, bulb-to-bulb distillation). MS *m/z*: 224 (M^+). HRMS Calcd for $C_{11}H_{19}F_3O$: 224.139. Found: 224.138. This product was found to be a mixture of two diastereoisomers (ratio 2 : 1), which were separated by a high performance liquid chromatography (HPLC) (Polygosil 60-5, 2-propanol–hexane, 1 : 6000). **13a** (major isomer): ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6.9 Hz), 1.20—1.50 (7H, m), 1.37 (3H, s), 1.60—1.70 (2H, m), 1.73—1.84 (1H, m), 1.97—2.06 (1H, m), 2.30 (1H, ddd, J=12.8, 8.2, 3.2 Hz), 4.01—4.10 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.82 (s). **13b**: ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz), 1.24—1.50 (7H, m), 1.36 (3H, s), 1.51—1.60 (1H, m), 1.60—1.70 (1H, m), 1.82 (1H, ddd, J=13.1, 8.2,

3.0 Hz), 2.02—2.11 (1H, m), 2.24 (1H, ddd, J=13.1, 8.2, 8.2 Hz), 4.01—4.10 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.65 (s). The two-dimensional NOE of **13a** showed a strong association between the 5-H and the 2-methyl protons, while that of **13b** showed associations between the 2-methyl and the 5-pentyl protons.

5-Heptyl-2-methyl-2-(trifluoromethyl)tetrahydrofuran (15)——A mixture of 1,1,1-trifluoro-2-methyl-4-dodecen-2-ol (**14**, 251 mg, 1.0 mmol) and p-TsOH·H_2O (140 mg, 0.7 mmol) in benzene (10 ml) was refluxed for 35 h. After the usual work-up of the reaction mixture, the residue was purified by column chromatography (SiO_2, CH_2Cl_2–hexane, 1:4) to give **15** (225 mg, 89%). **15**: colorless oil. MS m/z: 252 (M^+). HRMS Calcd for $C_{13}H_{23}F_3O$: 252.170. Found: 252.170. ^{1}H-NMR ($CDCl_3$) δ: 0.70—2.50 (19H, m), 1.37 (3H, s), 3.87—4.23 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.82 (s), 18.64 (s) (ratio 2:1). The ^{19}F-NMR spectrum and GLC analysis on a capillary column showed that **15** contained two diastereoisomers, but they could not be separated. From a comparison of the ^{19}F-NMR signals with those of **13a, b**, the major isomer seems to be the (2R*,5R*)-isomer.

(2R*,3R*,5S*)- (17a) and (2R*,3R*,5R*)-5-Butyl-2,3-dimethyl-2-(trifluoromethyl)tetrahydrofuran (17b)——A mixture of (2R*,3R*)-1,1,1-trifluoro-2,3-dimethyl-4-nonen-2-ol (**16**, 112 mg, 0.5 mmol) and p-TsOH·H_2O (95 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 34 h. After a usual work-up, the residue was passed through an SiO_2 column in hexane. The effluent gave **17** as a colorless oil, bp 70—75 °C (23 mmHg, bulb-to-bulb distillation). MS (CI) m/z: 225 (M+1). *Anal.* Calcd for $C_{11}H_{19}F_3O$: C, 58.91; H, 8.53. Found: C, 59.09; H, 8.71. This oil was found to be a mixture of two diastereoisomers, which were separated by medium-pressure column chromatography (SiO_2, hexane) to give **17a** (35 mg, 31%) and **17b** (11 mg, 10%). **17a**: colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz), 1.06 (3H, d, J=7.0 Hz), 1.20 (3H, d, J=1.0 Hz), 1.21—1.38 (5H, m), 1.38—1.50 (1H, m), 1.61—1.71 (1H, m), 2.13 (1H, ddd, J=12.0, 6.0, 5.0 Hz), 2.59 (1H, ddq, J=12.0, 7.0, 7.0 Hz), 3.90—4.00 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.66 (s). **17b**: colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7.0 Hz), 1.05 (3H, d, J=7.0 Hz), 1.22 (3H, q, J=1.0 Hz), 1.18—1.44 (5H, m), 1.53—1.65 (1H, m), 1.76 (1H, ddd, J=13.0, 8.0, 7.0 Hz), 1.90 (1H, dddq, J=13.0, 7.0, 5.0, 1.0 Hz), 2.56 (1H, ddq, J=7.0, 7.0, 7.0 Hz), 4.06—4.14 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 18.24 (s). The two-dimensional NOE showed a strong association between the protons of the 2-methyl and the 3-methyl group for both products, but an association between the 5-proton and those of the methyl group at 2-position was observed only for **17b**.

Attempted Cyclization of 1,1,1-Trifluoro-2-methyl-4-phenyl-4-penten-2-ol (18), Formation of 5,5,5-Trifluoro-4-methyl-2-phenyl-1,3-pentadiene (19)——A mixture of **18** (234 mg, 1.0 mmol) and p-TsOH·H_2O (95 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 12 h. After the usual work-up, the residue was purified by column chromatography (SiO_2, hexane) to give **19** (164 mg, 77%). **19**: colorless oil. MS m/z: 212 (M^+). HRMS Calcd for $C_{12}H_{11}F_3$: 212.081. Found: 212.080. ^{1}H-NMR ($CDCl_3$) δ: 1.82 (3H, d, J=1.5 Hz), 5.25 (1H, br s), 5.73 (1H, br s), 6.78 (1H, br s), 7.25—7.51 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 6.69 (s). This result shows that **19** is a single isomer.

(2R*,5S*)- (21a) and (2R*,5R*)-2-Butyl-5-propyl-2-(trifluoromethyl)tetrahydrofuran (21b)——A mixture of 5-(trifluoromethyl)-7-undecen-5-ol (**20**, 238 mg, 1.0 mmol) and p-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 50 h. After a usual work-up, the residue was separated by column chromatography (SiO_2, hexane–AcOEt, 100:1) to give **21** (190 mg, 80%). **21**: colorless oil, bp 90 °C (20 mmHg, bulb-to-bulb distillation). MS m/z: 237 (M−1). HRMS Calcd for $C_{12}H_{20}F_3O$ (M−H): 237.147. Found: 237.149. *Anal.* Calcd for $C_{12}H_{21}F_3O$: C, 60.48; H, 8.88. Found: C, 60.44; H, 9.14. The product was found by analysis of the ^{19}F-NMR spectrum to be a mixture of two diastereoisomers, which were separated by HPLC (SiO_2 in hexane) to **21a** and **21b** (eluted in that order). **21a**: ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, t, J=7.1 Hz), 0.93 (3H, t, J=7.1 Hz), 1.20—1.75 (11H, m), 1.86—1.94 (1H, m), 2.00—2.08 (1H, m), 2.15 (1H, ddd, J=13.0, 10.0, 8.5 Hz), 4.03—4.10 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 17.26 (s). **21b**: ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, t, J=7.2 Hz), 0.93 (3H, t, J=7.2 Hz), 1.25—1.50 (7H, m), 1.57—1.69 (3H, m), 1.70—1.80 (1H, m), 1.87—2.03 (2H, m), 2.12—2.20 (1H, m), 3.98—4.08 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 17.35 (s). The ratio of **21a** and **21b** was 1:3. The two-dimensional NOE of **21b** showed an association between the 5-H and the 2-butyl protons.

2-Butyl-5-pentyl-2-(trifluoromethyl)tetrahydrofuran (23)——A mixture of 5-(trifluoromethyl)-7-tridecen-5-ol (**22**, 269 mg, 1.0 mmol) and p-TsOH·H_2O (190 mg, 1.0 mmol) in benzene (10 ml) was refluxed for 41 h. After the usual work-up of the reaction mixture, the product was purified by column chromatography (SiO_2, CH_2Cl_2–hexane, 1:3) to give **23** (195 mg, 73%). **23**: colorless oil. MS m/z: 266 (M^+). HRMS Calcd for $C_{14}H_{25}F_3O$: 266.186. Found: 266.185. ^{1}H-NMR ($CDCl_3$) δ: 0.74—2.35 (24H, m), 3.83—4.26 (1H, m). ^{19}F-NMR ($CDCl_3$) ppm: 17.32 (s), 17.24 (s), (ratio 5:2). This shows that **23** is composed of two diastereoisomers. The main product is considered to be the (2R*,5R*)-isomer from a comparison of the ^{19}F-NMR spectra of **21** and **23**.

2-Butyl-5-phenyl-2-(trifluoromethyl)tetrahydrofuran (25)——A mixture of 1-phenyl-4-(trifluoromethyl)-1-octen-4-ol (**24**, 108 mg, 0.4 mmol) and p-TsOH·H_2O (95 mg, 0.5 mmol) in benzene (10 ml) was refluxed for 12 h. After the usual work-up of the reaction mixture, the residue was separated by medium-pressure column chromatography (SiO_2, CH_2Cl_2–hexane, 1:4) to give **25** (83 mg, 87%). **25**: colorless oil. MS m/z: 272 (M^+). HRMS Calcd for $C_{15}H_{19}F_3O$: 272.139. Found: 272.139. The product was found by GLC to be a mixture of two diastereoisomers (ratio 3:2), which were separated by medium-pressure column chromatography (same conditions as above). The former effluent; the minor product: ^{1}H-NMR ($CDCl_3$) δ: 0.75—2.59 (13H, m), 4.88—5.22 (1H, m), 7.06—7.68 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 16.79 (s). The major product: ^{1}H-NMR ($CDCl_3$) δ: 0.68—2.50 (13H, m), 4.78—5.18 (1H, m),

6.98—7.63 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 16.88 (s). The former isomer is considered to be the (2*R**,5*R**)-isomer, based on a comparison of the NMR data with those of **11**.

(2*R,5*S**)- (27a) and (2*R**,5*R**)-2-Phenyl-5-propyl-2-(trifluoromethyl)tetrahydrofuran (27b)**——A mixture of 1,1,1-trifluoro-2-phenyl-4-octen-2-ol (**26**, 156 mg, 0.6 mmol) and *p*-TsOH·H_2O (124 mg, 0.7 mmol) in benzene (10 ml) was refluxed for 35 h. After the usual work-up of the reaction mixture, the residue was purified by column chromatography (SiO_2, CH_2Cl_2–hexane, 1 : 5) to give **27** (117 mg, 75%). **27**: colorless oil, bp 115—120 °C (15 mmHg, bulb-to-bulb distillation). MS *m/z*: 258 (M^+). HRMS Calcd for $C_{14}H_{17}F_3O$: 258.123. Found: 258.123. This oil was found by analysis of the ^{19}F-NMR spectrum to be a mixture of two diastereoisomers (ratio 3 : 2), which were separated by HPLC using an ODS-C18 column in MeOH–H_2O (7 : 3) to give **27a** and **27b**. **27a**: ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, J=7.2 Hz), 1.32—1.55 (4H, m), 1.64—1.75 (1H, m), 2.09—2.18 (1H, m), 2.37 (1H, ddd, J=13.0, 8.0, 4.0 Hz), 2.64 (1H, ddd, J=13.0, 9.5, 8.0 Hz), 4.27—4.36 (1H, m), 7.28—7.40 (3H, m), 7.56 (2H, ddd, J=8.0, 2.0, 1.0 Hz). ^{19}F-NMR ($CDCl_3$) ppm: 15.78 (s). **27b**: ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, t, J=7.2 Hz), 1.35—1.62 (3H, m), 1.70—1.82 (2H, m), 1.93—2.02 (1H, m), 2.27 (1H, ddd, J=13.0, 8.5, 8.5 Hz), 2.73 (1H, ddd, J=13.0, 8.5, 4.0 Hz), 4.00—4.10 (1H, m), 7.28—7.40 (3H, m), 7.50—7.55 (2H, m). ^{19}F-NMR ($CDCl_3$) ppm: 16.28 (s). The two-dimensional NOE of **27b** shows an association between the 2-phenyl and the 5-propyl protons, while that of **27a** does not.

5-Pentyl-2-phenyl-2-(trifluoromethyl)tetrahydrofuran (29)——A mixture of 1,1,1-trifluoro-2-phenyl-4-decen-2-ol (**28**, 150 mg, 0.5 mmol) and *p*-TsOH·H_2O (127 mg, 0.7 mmol) in benzene (5 ml) was refluxed for 30 h. After the usual work-up of the reaction mixture, the residue was purified by column chromatography (SiO_2, hexane) to give **29** (134 mg, 89%). **29**: colorless oil. MS *m/z*: 286 (M^+). HRMS Calcd for $C_{16}H_{21}F_3O$: 286.154. Found: 286.155. This oil was found by GLC to be a mixture of two diastereoisomers (ratio 3 : 2), which were separated by medium-pressure column chromatography (SiO_2, hexane). The former effluent; the minor product: ^{1}H-NMR ($CDCl_3$) δ: 0.76—2.47 (14H, m), 2.58—2.91 (1H, m), 3.84—4.22 (1H, m), 7.20—7.70 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 16.22 (s). The major product: ^{1}H-NMR ($CDCl_3$) δ: 0.55—2.85 (15H, m), 3.98—4.47 (1H, m), 7.12—7.69 (5H, m). ^{19}F-NMR ($CDCl_3$) ppm: 15.75 (s). The latter is considered to be the (2*R**,5*S**)-isomer from a comparison of its NMR data with those of **27**.

References and Notes

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5) Molecular mechanics calculations were carried out according to the method presented by E. Osawa and Y. Mochizuki, *Kagaku*, **40**, 703 (1985), using an MM2 program modified by them for use on a personal computer. The calculations were performed on PC-9801F2 (NEC, Japan).

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Quinolizidines. XXII.[1] An Extension of the "3-Acetylpyridine Route" to the Syntheses of 9-Hydroxy-10-methoxy- and 10-Hydroxy-9-methoxybenzo[*a*]quinolizidine-Type *Alangium* Alkaloids

TOZO FUJII,* MASASHI OHBA, and JUN SAKAGUCHI

Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi, Kanazawa 920, Japan

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Alternative syntheses of the *Alangium* alkaloids bearing the 9-hydroxy-10-methoxy- and 10-hydroxy-9-methoxybenzo[*a*]quinolizidine skeletons (types **3** and **4**) have now become feasible through generally applicable routes starting from 3-acetylpyridine. The routes include the mercuric acetate–edetic acid oxidation of the 3-acetylpyridine derivatives **8a**, **b** or the alkaline ferricyanide oxidation of the quaternary salts (**29a**, **b** and **30a**) of 3-acetylpyridine equivalents, Wolff–Kishner reduction of the acetyl group or reductive desulfurization of the thioketal group, sulfenylation–dehydrosulfenylation of the lactams **15a**, **b**, Michael reaction of the α,β-unsaturated lactams **18a**, **b**, and hydrolysis, decarboxylation, and esterification of the Michael adducts to give **21a**, **b**, as the main operations.

Keywords——*Alangium* alkaloid synthesis; piperidine mercuric acetate–edetic acid oxidation; lactam sulfenylation–dehydrosulfenylation; α,β-unsaturated lactam Michael reaction; malonic acid decarboxylation; pyridinium salt ferricyanide oxidation; thioketal reductive desulfurization; *O*-benzyl group hydrogenolysis; reductive demethoxylation

So far, 18 benzo[*a*]quinolizidine alkaloids[2,3] and 14 other alkaloids[2,4] have been found in various parts of the Indian medicinal plant *Alangium lamarckii* THWAITES (Alangiaceae). These 18 benzo[*a*]quinolizidine-type *Alangium* alkaloids may be structurally classified into four groups according to their substitution patterns in the aromatic ring A[5]: (i) 9,10-dimethoxy type (**1**), (ii) 8-hydroxy-9,10-dimethoxy type (**2**), (iii) 9-hydroxy-10-methoxy type (**3**), and (iv) 10-hydroxy-9-methoxy type (**4**).[6] Our previous studies have shown that the racemic syntheses of all of these types of alkaloids are possible through the "lactim ether route"[7] and the chiral syntheses, through the "cincholoipon-incorporating route"[7] or "lactim ether route."[7] Moreover, the recently developed "3-acetylpyridine route" has been shown to be applicable to the racemic syntheses of **1**- and **2**-type alkaloids.[5] In the present study, an extension of this "3-acetylpyridine route" to the syntheses of the remaining **3**- and **4**-type *Alangium* alkaloids was accomplished by adopting the following two tactics.[8]

1 **2** **3** **4**

R = CH_2OH, CO_2H, or a heterocyclic ring

The Route through Mercuric Acetate–Edetic Acid [$Hg(OAc)_2$–EDTA] Oxidation

The synthesis of the 3-type alkaloids was initiated by quaternization of the ketal **5**, a 3-acetylpyridine equivalent, with 3-benzyloxy-4-methoxyphenacyl bromide in benzene at room temperature. The resulting quaternary salt **6a** (92% yield) was reduced successively with hydrogen over Adams catalyst and with $NaBH_4$ to furnish the amino alcohol **7a** in 72% yield as a diastereomeric mixture. Deketalization of **7a** with 1 N aqueous HCl at 40 °C gave the ketone **8a** (98% yield), which was oxidized with $Hg(OAc)_2$–EDTA according to the previously reported standard procedure,[9] producing the 6-piperidone **10a** (as a diastereomeric mixture) and the enamine **9a** (a 2-oxidation product[10]) in 76% and 8% yields, respectively. The ratio of the 6- to the 2-oxidation in this case was 90 : 10, being comparable to that observed[5] by us for

a : R^1 = CH_2Ph ; R^2 = Me

b : R^1 = Me; R^2 = CH_2Ph

Chart 1

similar oxidations of the 9,10-dimethoxy and 8-benzyloxy-9,10-dimethoxy analogues. The diastereomeric mixture **10a** was then hydrogenolyzed over 10% Pd–C using hydrogen in the presence of perchloric acid, and the resulting phenolic ketone **11** (71% yield) was subjected to the Huang–Minlon modification of the Wolff–Kishner reduction, affording the phenolic lactam **13** in 84% yield. Benzylation of **13** with benzyl bromide in boiling acetone containing K_2CO_3 gave the benzyl ether **15a** in 97% yield. On sulfenylation[11] with diphenyl disulfide in tetrahydrofuran (THF) in the presence of lithium diisopropylamide (LDA) and hexamethylphosphoramide (HMPA) at −78 °C, **15a** produced the sulfide **16a** in 77% yield as a diastereomeric mixture. The mixture **16a** was then oxidized with sodium metaperiodate in aqueous MeOH, and thermolysis of the resulting sulfoxide **17a** in boiling toluene in the presence of $CaCO_3$ provided the α,β-unsaturated lactam **18a** in 82% yield (from **16a**), thus terminating the sulfenylation–dehydrosulfenylation of **15a**. This sulfenylation–dehydrosulfenylation of the six-membered lactam has a few precedents in the literature.[5,12] The infrared (IR) spectrum of **18a** in $CHCl_3$ at 0.2 M concentration showed two strong absorptions at 1660 and 1605 cm^{-1}, which shifted to 1648 and 1576 cm^{-1}, respectively, on addition of an equimolar amount of dichloroacetic acid. This permitted us to assign the 1660 cm^{-1} band to the C=C stretching vibration and the 1605 cm^{-1} band to the CO stretching vibration in the six-membered, α,β-unsaturated lactam system.[13]

The Michael acceptor **18a** was then allowed to react with diethyl malonate in a manner similar to that reported[5,14] previously,[15] and the resulting adduct was hydrolyzed with 1 N aqueous NaOH in EtOH to give **19a** (presumed to be a mixture of the *trans* and *cis* isomers) in 51% yield (from **18a**). On treatment with boiling 60% aqueous AcOH for 6 h, **19a** was decarboxylated to give a crude acid (99% yield), which was shown to be an 83 : 17 mixture of the *trans* isomer **20a** and its *cis* isomer on carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopic analysis. Purification of the crude acid by recrystallization furnished the *trans*-lactam acid **20a** (59% yield from **19a**), and esterification of **20a** with ethanolic HCl at room temperature gave the *trans*-lactam ester **21a** in 91% yield. The structure and stereochemistry of **21a** thus obtained were confirmed by its identity with an authentic sample prepared through the "lactim ether route."[16]

Since the lactam ester **21a** has already been converted by us into (±)-9-demethylpsychotrine,[16] (±)-9-demethyltubulosine,[17] and (±)-9-demethylprotoemetinol,[18] the above synthesis of **21a** from the ketal **5** formally concludes new racemic syntheses of these three *Alangium* alkaloids bearing the 9-hydroxy-10-methoxybenzo[*a*]quinolizidine skeleton (type **3**).

For the synthesis of the **4**-type alkaloids, a parallel sequence of conversions was then investigated. It started with quaternization of **5** with 4-benzyloxy-3-methoxyphenacyl bromide to give **6b** (91% yield), proceeded through the intermediates **7b** (72%),[19] **8b** (95%),[19] **10b** (80%)[19] [with **9b** (8%)], **12** (95%), **14** (92%), **15b** (94%), **16b** (70%),[19] **17b**, **18b** (67% from **16b**), **19b** (61%) (as a mixture of the *trans* and *cis* isomers), and **20b** (65% after recrystallization of a 90 : 10 mixture of the *trans* and *cis* isomers obtained from **19b** by decarboxylation), and terminated at **21b** (89% yield). The *trans*-lactam ester **21b** thus prepared was identical with an authentic sample synthesized through the "lactim ether route."[16] The assignment of the two strong absorption bands at 1660 (C=C) and 1603 cm^{-1} (CO) observed in the IR spectrum of **18b** in $CHCl_3$ was based on a band-shift experiment similar to that employed for **18a**.

In view of the previous conversions of **21b** into (±)-10-demethylpsychotrine,[16,20] (±)-10-demethyltubulosine,[21] and (±)-10-demethylprotoemetinol,[18] the above synthesis of **21b** from the ketal **5** formally amounts to new syntheses of these two *Alangium* alkaloids and a related structure, which all possess the 10-hydroxy-9-methoxybenzo[*a*]quinolizidine skeleton (type **4**).

The Route through Alkaline Ferricyanide Oxidation

The requisite starting materials for this route were the phenethyl bromides **27a**, **b**, and they were prepared by the following 5-step synthesis. Willgerodt–Kindler reaction of the ketone **22a** and alkaline hydrolysis of the resulting thiomorpholide **23a**, reported by Inubushi *et al.*,[22] were effected with a slight modification, giving the carboxylic acid **24a** in 59% overall yield. On esterification with ethanolic HCl, **24a** produced the ester **25a** (94% yield), which was then reduced with $LiAlH_4$ in ether to afford the alcohol **26a** in 97% yield. Bromination of **26a** with *N*-bromosuccinimide/Ph_3P reagent[23] in benzene furnished the desired bromide **27a** in 93% yield. A parallel series of conversions starting with the isomeric ketone **22b** gave **24b** (60% yield *via* **23b**), **25b** (94%), **26b** (96%), and **27b** (92%).

Quaternizations of the ketal **5** with the bromides **27a** and **27b** and that of the thioketal **28** with **27a** in boiling benzene afforded the pyridinium salts **29a**, **29b**, and **30a**. The alkaline ferricyanide oxidations of **29a**, **29b**, and **30a** were separately carried out under the standard

a: R^1=CH₂Ph; R^2=Me

b: R^1=Me; R^2=CH₂Ph

Chart 2

conditions described previously,[9a,24] providing the 6-oxidation products **31a** (85% overall yield from **5**), **31b** (86% from **5**), and **32a** (67% from **28**), respectively. As in the previous results with analogous pyridinium salts,[2b,5,24] no 2-oxidation products were obtained in these three oxidations. Catalytic hydrogenations of the pyridones **31a** and **31b** over Raney Ni catalyst in EtOH at atmospheric pressure and room temperature produced the phenolic lactams **33** and **34** in 97% and 99% yields, respectively. Such a facile hydrogenolysis of the *O*-benzyl group over Raney Ni catalyst has also been observed for the *N*-(2-benzyloxy-3,4-dimethoxyphenethyl) analogue.[5] Finally, deketalizations of **33** and **34** with 10% aqueous HCl in EtOH gave the lactam ketones **11** and **12** in 96% and 98% yields, respectively.

In much the same sense, the pyridone **32a** was subjected to reductive desulfurization using Raney Ni in boiling aqueous EtOH for 1 h, giving the phenolic lactam **13** in 66% yield. Interestingly, the demethoxylated compound **36** was a by-product (20% yield) in this reaction. The observed demethoxylation is rather unusual, but a few precedents have been found in the literature.[25] The structure of **36** was confirmed by an alternative synthesis, which furnished **36** from the known lactam **35**[19,26] in 94% yield by catalytic hydrogenolysis.

Since the phenolic lactams **11**, **12**, and **13** have already been led to the **3**- and **4**-type *Alangium* alkaloids by the routes shown in Chart 1, the above syntheses of **11**, **12**, and **13** from **5** and **28** adopting the alkaline ferricyanide oxidation tactics are tantamount to additional new formal syntheses of these alkaloids.

Conclusion

The present work has revealed that the **3**- and **4**-types of *Alangium* alkaloids can be synthesized either from the ketal **5** (a 3-acetylpyridine equivalent) by utilizing $Hg(OAc)_2$–EDTA oxidation or from **5** or the thioketal **28** (another 3-acetylpyridine equivalent) by employing alkaline ferricyanide oxidation. The latter tactic in this "3-acetylpyridine strategy" is based on the early idea of Professor Sugasawa, who attempted to utilize the alkaline ferricyanide oxidation of 1-substituted 3-ethylpyridinium salt or its equivalents for synthesizing the ipecac alkaloid emetine (type **1**) in the early 1950's.[27]

In summary, it should be emphasized that the "3-acetylpyridine route" has now become applicable to the racemic syntheses of all of the benzo[*a*]quinolizidine-type *Alangium* alkaloids (**1**—**4**) as a result of our previous[5] and present studies. This extension has made its applicability comparable to that of the already established "lactim ether route"[7] and to that of the "cincholoipon-incorporating route"[7] in the chiral series.

Experimental

General Notes——All melting points were determined by using a Yamato MP-1 capillary melting point apparatus and are corrected. Unless otherwise stated, the organic solutions obtained after extraction were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. See ref. 1 for details of instrumentation and measurements. Solution IR spectra were measured in $CHCl_3$ solutions at 0.2 M concentration. Elemental analyses 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, dt = doublet-of-triplets, m = multiplet, q = quartet, s = singlet, sh = shoulder, t = triplet.

1-(3-Benzyloxy-4-methoxyphenacyl)-3-(1,1-ethylenedioxyethyl)pyridinium Bromide (6a)——A solution of **5**[27c] (2.73 g, 16.5 mmol) and 3-benzyloxy-4-methoxyphenacyl bromide[28] (5.03 g, 15 mmol) in dry benzene (35 ml) was stirred at room temperature for 24 h. The precipitate that resulted was filtered off, washed with benzene, and dried to give a first crop of **6a**. The filtrate and washings were combined, concentrated to a small volume, and stirred at room temperature for 4 h to yield a second crop of **6a**. The first and second crops of **6a** were combined and recrystallized from H_2O–EtOH (3 : 1, v/v) to give **6a**·$3/2H_2O$ (7.29 g, 92%) as yellowish prisms. Further recrystallization from the same solvent and drying over P_2O_5 at 2 mmHg and room temperature for 22 h afforded an analytical sample as slightly yellowish prisms, mp 83—87 °C; IR ν_{max}^{Nujol} cm^{-1}: 3420 (H_2O), 1682 (CO); ^{1}H-NMR ($CDCl_3$) δ: 1.73 (3H, s, CMe), 2.13 (3H, s, H_2O), 3.90 (3H, s, OMe), 3.8—4.2 (4H, m, OCH_2's), 5.20 (2H, s, $OC\underline{H}_2Ph$), 6.93 (1H, d, J= 8.5 Hz, $H_{(5')}$), 7.10 (2H, s, $ArCOC\underline{H}_2$), 7.2—7.55 (5H, m, Ph), 7.65 (1H, d, J=2 Hz, $H_{(2')}$), 7.96 (1H, dd, J=8.5, 2 Hz,

$H_{(6')}$), 8.00 (1H, dd, J=8, 6 Hz, $H_{(5)}$), 8.45 (1H, d, J=8 Hz, $H_{(4)}$), 8.91 (1H, s, $H_{(2)}$), 9.47 (1H, d, J=6 Hz, $H_{(6)}$).[29] *Anal.* Calcd for $C_{25}H_{26}BrNO_5 \cdot 3/2H_2O$: C, 56.93; H, 5.54; N, 2.66. Found: C, 56.99; H, 5.47; N, 2.70.

1-(4-Benzyloxy-3-methoxyphenacyl)-3-(1,1-ethylenedioxyethyl)pyridinium Bromide (6b)——A stirred solution of 5[27c] (3.47 g, 21 mmol) and 4-benzyloxy-3-methoxyphenacyl bromide[30] (7.04 g, 21 mmol) in dry benzene (30 ml) was heated under reflux for 5 min. The precipitate that resulted was filtered off and washed with benzene to give a first crop of **6b**. The filtrate and washings were combined, concentrated to a small volume, and heated under reflux for 25 min to yield a second crop of **6b**. A third crop was further obtained by a similar treatment (reflux, 4.5 h) of the mother liquor. Recrystallization of the first, second, and third crops of **6b** from H_2O–EtOH (3:1, v/v) afforded **6b**$\cdot 1/3H_2O$ (9.69 g, 91%). Further recrystallization from the same solvent and drying over P_2O_5 at 2 mmHg and room temperature for 41 h and then at 50 °C for 16 h yielded an analytical sample as yellowish orange needles, mp 86—95 °C (dec.); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400 (H_2O), 1682 (CO); ^{1}H-NMR ($CDCl_3$) δ: 1.74 (3H, s, CMe), 1.85 (0.7H, s, H_2O), 3.94 (3H, s, OMe), 3.8—4.2 (4H, m, OCH_2's), 5.20 (2H, s, $OC\underline{H}_2Ph$), 6.95 (1H, d, J=8.5 Hz, $H_{(5')}$), 7.13 (2H, s, $ArCOC\underline{H}_2$), 7.2—7.5 (5H, m, Ph), 7.62 (1H, d, J=2 Hz, $H_{(2')}$), 7.87 (1H, dd, J=8.5, 2 Hz, $H_{(6')}$), 7.99 (1H, dd, J=8, 6 Hz, $H_{(5)}$), 8.47 (1H, dt, J=8, 1.5 Hz, $H_{(4)}$), 8.90 (1H, br, $H_{(2)}$), 9.54 (1H, dt, J=6, 1.5 Hz, $H_{(6)}$).[29] *Anal.* Calcd for $C_{25}H_{26}BrNO_5 \cdot 1/3H_2O$: C, 59.30; H, 5.31; N, 2.77. Found: C, 59.31; H, 5.14; N, 2.80.

1-(3-Benzyloxy-4-methoxyphenyl)-2-[3-(1,1-ethylenedioxyethyl)piperidino]ethanol (7a)——A solution of **6a**$\cdot 3/2H_2O$ (7.12 g, 13.5 mmol) in 50% aqueous EtOH (60 ml) was hydrogenated over Adams catalyst (200 mg) at atmospheric pressure and room temperature for 16 h, taking up *ca.* 3 molar eq of H_2. The catalyst was removed by filtration, and 1 N aqueous NaOH (13.5 ml) and $NaBH_4$ (511 mg, 13.5 mmol) were added to the filtrate. The resulting mixture was stirred at room temperature for 24 h and then concentrated *in vacuo*. The residue was partitioned by extraction with a mixture of H_2O and benzene. The benzene extracts were washed with H_2O, dried over anhydrous K_2CO_3, and concentrated to leave a yellow oil (5.38 g). Purification of the oil by column chromatography [alumina, hexane–AcOEt (4:1, v/v)] afforded **7a** (4.17 g, 72%) as a faintly yellowish oil,[19] MS m/z: 427 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420 (OH); ^{1}H-NMR ($CDCl_3$) δ: 1.25 and 1.27 (3H, s each, diastereomeric CMe's), 3.86 (3H, s, OMe), 3.8—4.0 (4H, m, OCH_2's), 4.5—4.75 [1H, m, $ArC\underline{H}(OH)$], 5.14 (2H, s, $OC\underline{H}_2Ph$), 6.75—7.05 (3H, m, aromatic protons), 7.2—7.55 (5H, m, Ph).

1-(4-Benzyloxy-3-methoxyphenyl)-2-[3-(1,1-ethylenedioxyethyl)piperidino]ethanol (7b)——This was prepared from **6b**$\cdot 1/3H_2O$ in 72% yield in a manner similar to that described above for **7a** and isolated as a colorless oil,[19] MS m/z: 427 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400 (OH); ^{1}H-NMR ($CDCl_3$) δ: 1.25 and 1.27 (3H, s each, diastereomeric CMe's), 3.8—4.0 (4H, m, OCH_2's), 3.90 (3H, s, OMe), 4.5—4.75 [1H, m, $ArC\underline{H}(OH)$], 5.13 (2H, s, $OC\underline{H}_2Ph$), 6.7—7.0 (3H, m, aromatic protons), 7.15—7.5 (5H, m, Ph).

1-[2-(3-Benzyloxy-4-methoxyphenyl)-2-hydroxyethyl]-3-piperidyl Methyl Ketone (8a)——A mixture of **7a** (1.71 g, 4 mmol) and 1 N aqueous HCl (12 ml) was stirred at 40 °C for 2.5 h. The resulting solution was basified (pH 10) with K_2CO_3 and extracted with benzene. The benzene extracts were washed with saturated aqueous NaCl, dried (K_2CO_3), and concentrated to leave **8a** (1.50 g, 98%) as a faintly yellowish oil,[19] MS m/z: 383 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3420 (OH), 1707 (CO); ^{1}H-NMR ($CDCl_3$) δ: 2.16 and 2.18 (3H, s each, diastereomeric COMe's), 3.86 (3H, s, OMe), 4.5—4.7 [1H, m, $ArC\underline{H}(OH)$], 5.14 (2H, s, $OC\underline{H}_2Ph$), 6.75—7.0 (3H, m, aromatic protons), 7.2—7.55 (5H, m, Ph).

1-[2-(4-Benzyloxy-3-methoxyphenyl)-2-hydroxyethyl]-3-piperidyl Methyl Ketone (8b)——This was prepared in 95% yield from **7b** by deketalization similar to that described above for **8a**. Purification by means of column chromatography [alumina, hexane–AcOEt (2:1, v/v)] yielded **8b** as a colorless oil,[19] MS m/z: 383 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3410 (OH), 1705 (CO); ^{1}H-NMR ($CDCl_3$) δ: 2.17 and 2.19 (3H, s each, diastereomeric COMe's), 3.90 (3H, s, OMe), 4.55—4.75 [1H, m, $ArC\underline{H}(OH)$], 5.14 (2H, s, $OC\underline{H}_2Ph$), 6.7—7.0 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph).

The $Hg(OAc)_2$–EDTA Oxidation of 8a and 8b——The oxidations of **8a** and **8b** with $Hg(OAc)_2$–EDTA were carried out in boiling 1% aqueous AcOH for 1.5 h according to the previously reported procedure.[9] Work-up of the reaction mixtures also followed that procedure, giving the 6-piperidone **10a** (76% yield) and the enamine **9a** (8%) from **8a**; **10b** (80%) and **9b** (8%) from **8b**. Separation of the two products in each case was achieved by means of column chromatography using silica gel and $CHCl_3$ or $CHCl_3$–MeOH (50:1, v/v), and the 6-piperidones (**10a, b**) were eluted faster than the enamines (**9a, b**). These products were characterized as described below.

1-[2-(3-Benzyloxy-4-methoxyphenyl)-2-hydroxyethyl]-1,2,3,4-tetrahydro-5-pyridyl Methyl Ketone (9a)——This enamine was recrystallized from AcOEt to yield pale yellowish granules, mp 122.5—124 °C; MS m/z: 381 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3300 (OH), 1621 and 1570 (vinylogous amide)[31]; ^{1}H-NMR ($CDCl_3$) δ: 1.6—1.9 (2H, m, $H_{(3)}$'s), 2.04 (3H, s, COMe), 2.24 (2H, t, J=6 Hz, $H_{(4)}$'s), 2.8 (1H, br, OH), 3.08 (2H, t, J=5.5 Hz, $H_{(2)}$'s), 3.26 [2H, d, J=6 Hz, $ArCH(OH)C\underline{H}_2$], 3.87 (3H, s, OMe), 4.77 [1H, t, J=6 Hz, $ArC\underline{H}(OH)CH_2$], 5.14 (2H, s, $OC\underline{H}_2Ph$), 6.8—7.0 (3H, m, aromatic protons), 7.16 (1H, s, $H_{(6)}$), 7.2—7.55 (5H, m, Ph). *Anal.* Calcd for $C_{23}H_{27}NO_4$: C, 72.42; H, 7.13; N, 3.67. Found: C, 72.29; H, 7.18; N, 3.51.

1-[2-(4-Benzyloxy-3-methoxyphenyl)-2-hydroxyethyl]-1,2,3,4-tetrahydro-5-pyridyl Methyl Ketone (9b)——This was obtained as a yellow oil, MS m/z: 381 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3320 (OH), 1621 and 1570 (vinylogous amide)[31]; ^{1}H-NMR ($CDCl_3$) δ: 1.6—1.9 (2H, m, $H_{(3)}$'s), 2.06 (3H, s, COMe), 2.27 (2H, t, J=6 Hz, $H_{(4)}$'s), 2.55 (1H, br, OH), 3.16 (2H, t, J=5.5 Hz, $H_{(2)}$'s), 3.34 [2H, d, J=6 Hz, $ArCH(OH)C\underline{H}_2$], 3.89 (3H, s, OMe), 4.82 [1H, t, J=6 Hz,

ArC$\underline{H}$(OH)CH_2], 5.14 (2H, s, OC$\underline{H}_2$Ph), 6.7—7.0 (3H, m, aromatic protons), 7.24 (1H, s, $H_{(6)}$), 7.2—7.5 (5H, m, Ph).

5-Acetyl-1-[2-(3-benzyloxy-4-methoxyphenyl)-2-hydroxyethyl]-2-piperidone (10a)——This diastereomeric mixture was obtained as a pale yellowish solid, mp 125—131 °C; MS *m/z*: 397 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3370 (OH), 1715 (CO), 1621 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 2.09 and 2.18 (3H, s each, diastereomeric COMe's), 3.87 (3H, s, OMe), 4.8—5.0 [1H, m, ArC$\underline{H}$(OH)], 5.15 and 5.17 (2H, s each, diastereomeric OC$\underline{H}_2$Ph's), 6.75—7.05 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph).

5-Acetyl-1-[2-(4-benzyloxy-3-methoxyphenyl)-2-hydroxyethyl]-2-piperidone (10b)——This was isolated as a yellow oil,[19] MS *m/z*: 397 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400 (OH), 1713 (CO), 1623 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 2.13 and 2.20 (3H, s each, diastereomeric COMe's), 3.90 (3H, s, OMe), 4.8—5.0 [1H, m, ArC$\underline{H}$(OH)], 5.13 (2H, s, OC$\underline{H}_2$Ph), 6.7—7.0 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph).

5-Acetyl-1-(3-hydroxy-4-methoxyphenethyl)-2-piperidone (11)——A solution of **10a** (2.39 g, 6 mmol) in EtOH (80 ml) containing 70% aqueous $HClO_4$ (1.2 ml) was hydrogenated over 10% Pd–C (1.8 g) at 3.0—3.75 atm and 35 °C for 30 h. An additional amount (500 mg) of the catalyst was added at that point, and the mixture was further hydrogenated for 12 h. Removal of the catalyst by filtration and concentration of the filtrate under reduced pressure left an oil, which was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ extracts were washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, dried, and concentrated to leave a pale brownish oil. The oil was crystallized from hexane–AcOEt to give a first crop (919 mg) of **11** as colorless needles, mp 97—98 °C. Concentration of the mother liquor of this crystallization and column chromatographic purification (silica gel, $CHCl_3$) of the residue yielded a second crop of a colorless solid, mp 97.5—99 °C. The total yield was 1.25 g (71%). Recrystallization of the solid from hexane–AcOEt (1 : 2, v/v) gave an analytical sample of **11** as colorless needles, mp 99.5—100 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1715 (CO), 1631 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 2.16 (3H, s, COMe), 3.87 (3H, s, OMe), 5.70 (1H, br, OH), 6.6—6.9 (3H, m, aromatic protons). *Anal.* Calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.26; N, 4.81. Found: C, 66.02; H, 7.40; N, 4.78.

5-Acetyl-1-(4-hydroxy-3-methoxyphenethyl)-2-piperidone (12)——A solution of **10b** (1.99 g, 5 mmol) in EtOH (50 ml) containing 70% aqueous $HClO_4$ (1 ml) was hydrogenated over 10% Pd–C (1.50 g) at atmospheric pressure and room temperature for 5 h. The reaction mixture was worked up in a manner similar to that described above for **11**, giving **12** (1.39 g, 95%) as a slightly brownish solid, mp 115.5—116.5 °C. Recrystallization of the solid from benzene–hexane (3 : 1, v/v) produced an analytical sample as colorless prisms, mp 117—117.5 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1715 (CO), 1631 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, s, COMe), 3.88 (3H, s, OMe), 4.90 (1H, br, OH), 6.6—6.9 (3H, m, aromatic protons). *Anal.* Calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.26; N, 4.81. Found: C, 66.11; H, 7.38; N, 4.68.

5-Ethyl-1-(3-hydroxy-4-methoxyphenethyl)-2-piperidone (13)——A mixture of **11** (1.31 g, 4.5 mmol), ethylene glycol (9 ml), 80% aqueous hydrazine hydrate (563 mg, 9 mmol), and KOH (900 mg) was placed in a flask equipped with a descending condenser. The mixture was heated in an oil bath at 120 °C for 1 h with stirring. Then, the temperature of the oil bath was slowly raised to 190 °C in 30 min, and the mixture was further heated with stirring at 190—195 °C for 3 h to give a small amount of distillate. After cooling, the reaction mixture was poured into H_2O (100 ml), and the resulting solution was brought to pH 2 by addition of 10% aqueous HCl and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with saturated aqueous NaCl, dried, and concentrated to leave **13** (1.05 g, 84%) as a pale brownish solid, mp 115.5—118 °C. Recrystallization from hexane–AcOEt (1 : 2, v/v) yielded an analytical sample as colorless prisms, mp 118.5—119.5 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1625 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7 Hz, $CCH_2\underline{Me}$), 3.86 (3H, s, OMe), 6.6—6.9 (3H, m, aromatic protons). *Anal.* Calcd for $C_{16}H_{23}NO_3$: C, 69.29; H, 8.36; N, 5.05. Found: C, 69.44; H, 8.40; N, 5.15.

5-Ethyl-1-(4-hydroxy-3-methoxyphenethyl)-2-piperidone (14)——A mixture of **12** (8.74 g, 30 mmol), ethylene glycol (60 ml), 80% aqueous hydrazine hydrate (3.76 g, 60 mmol), and KOH (6.0 g) was allowed to react as described above for **13**, and crude **14** (7.62 g, 92%) was isolated as a pale brownish solid, mp 88—88.5 °C. Recrystallization from hexane–AcOEt (2 : 1, v/v) gave an analytical sample as colorless needles, mp 90.5—91.5 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1623 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7 Hz, $CCH_2\underline{Me}$), 3.88 (3H, s, OMe), 6.6—6.9 (3H, m, aromatic protons). *Anal.* Calcd for $C_{16}H_{23}NO_3$: C, 69.29; H, 8.36; N, 5.05. Found: C, 69.28; H, 8.38; N, 5.07.

1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-2-piperidone (15a)——A stirred mixture of **13** (3.33 g, 12 mmol) and benzyl bromide (2.46 g, 14.4 mmol) in acetone (50 ml) containing anhydrous K_2CO_3 (1.99 g, 14.4 mmol) was heated under reflux for 30 h. After cooling, the solvent was removed from the reaction mixture by vacuum distillation, and the residue was partitioned by extraction with a mixture of benzene and H_2O. The benzene extracts were washed successively with H_2O, 5% aqueous NaOH, and H_2O, dried, and concentrated to leave a faintly yellow oil. In order to remove the excess benzyl bromide, the oil was dissolved in benzene (30 ml) containing pyridine (6 ml), and the resulting solution was stirred at room temperature overnight. The precipitate that resulted was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was dissolved in benzene (200 ml), and the benzene solution was washed successively with H_2O, 5% aqueous HCl, and saturated aqueous NaCl, dried, and concentrated to leave a pale yellow oil. This material was purified by column chromatography [silica gel, hexane–AcOEt (1 : 1, v/v)] to give **15a** (4.26 g, 97%) as a colorless solid, mp 47.5—49.5 °C. Recrystallization from benzene–hexane (1 : 5, v/v) produced an analytical sample as colorless needles, mp 53—54.5 °C; MS *m/z*: 367 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1625 (lactam CO); ^{1}H-

NMR ($CDCl_3$) δ: 0.86 (3H, t, J=7Hz, $CH_2\underline{Me}$), 3.86 (3H, s, OMe), 5.14 (2H, s, $OC\underline{H}_2Ph$), 6.6—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{23}H_{29}NO_3$: C, 75.17; H, 7.95; N, 3.81. Found: C, 75.19; H, 8.09; N, 3.68.

1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-2-piperidone (15b)——A stirred mixture of **14** (6.66 g, 24 mmol) and benzyl bromide (4.93 g, 28.8 mmol) in acetone (120 ml) containing anhydrous K_2CO_3 (3.98 g, 28.8 mmol) was heated under reflux for 24 h. The reaction mixture was worked up in a manner similar to that described above for **15a**, giving **15b** (8.25 g, 94%) as a faintly yellow oil, MS m/z: 367 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1625 (lactam CO); ^1H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7Hz, $CH_2\underline{Me}$), 3.88 (3H, s, OMe), 5.12 (2H, s, $OC\underline{H}_2Ph$), 6.55—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph).

1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-3-phenylthio-2-piperidone (16a)——A stirred solution of diisopropylamine (1.1 ml, 7.8 mmol) in dry THF (8 ml) was cooled to $-78\,^{\circ}C$ in an atmosphere of N_2, and 1.34 M solution (4.5 ml, 6 mmol) of butyllithium in hexane was added dropwise. After the mixture had been stirred at the same temperature for 30 min, a solution of **15a** (1.11 g, 3 mmol) in THF (2 ml) was added dropwise over 10 min, and stirring was continued for 30 min. Next, a solution of diphenyl disulfide (659 mg, 3 mmol) and HMPA (0.52 ml, 3 mmol) in dry THF (2 ml) was added dropwise in 5 min. The resulting mixture was further stirred at $-78\,^{\circ}C$ for 2 h, brought to room temperature after addition of saturated aqueous NH_4Cl (4 ml), and extracted with benzene. The benzene extracts were washed with saturated aqueous NaCl, dried, and concentrated to leave a yellow oil (1.71 g). Purification of the oil by column chromatography [alumina, hexane–AcOEt (3 : 1, v/v)] provided **16a** (1.11 g, 77%) as a faintly yellow oil,[19] MS m/z: 475 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1632 and 1639 (sh) (diastereomeric lactam CO's); ^1H-NMR ($CDCl_3$) δ: 0.79 and 0.84 (3H, t each, J=7Hz, diastereomeric $CH_2\underline{Me}$'s), 3.86 (3H, s, OMe), 5.14 and 5.16 (2H, s each, diastereomeric $OC\underline{H}_2Ph$'s), 6.6—6.9 (3H, m, aromatic protons), 7.1—7.65 (10H, m, Ph's).

1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-3-phenylthio-2-piperidone (16b)——Sulfenylation of **15b** was carried out as described above for **16a**, and the crude oily product was purified on an alumina column [hexane–AcOEt (5 : 1, v/v)] to furnish **16b** in 70% yield as a yellow oil,[19] MS m/z: 475 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1632 and 1639 (sh) (diastereomeric lactam CO's); ^1H-NMR ($CDCl_3$) δ: 0.81 and 0.84 (3H, t each, J=7Hz, diastereomeric $CH_2\underline{Me}$'s), 3.88 and 3.90 (3H, s each, diastereomeric OMe's), 5.12 (2H, s, $OC\underline{H}_2Ph$), 6.5—6.9 (3H, m, aromatic protons), 7.1—7.6 (10H, m, Ph's).

1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-5,6-dihydro-2(1*H*)-pyridinone (18a)——A stirred solution of **16a** (1.00 g, 2.1 mmol) in MeOH (20 ml) was cooled in an ice bath, and a solution of $NaIO_4$ (492 mg, 2.3 mmol) in H_2O (5 ml) was added dropwise over 5 min. The mixture was stirred at room temperature for 24 h, and the insoluble material that resulted was filtered off and washed with MeOH (20 ml). The filtrate and washings were combined and concentrated *in vacuo*, and the residue was dissolved in benzene (80 ml). The benzene solution was washed with saturated aqueous NaCl, dried, and concentrated to leave the sulfoxide **17a** (1.17 g) as a yellow oil. A solution of this oil in toluene (40 ml) containing $CaCO_3$ (1.00 g, 10 mmol) was then heated under reflux in an atmosphere of N_2 for 1 h. The reaction mixture was filtered in order to remove the insoluble solid, and the filtrate was washed with saturated aqueous NaCl, dried, and concentrated to leave a yellowish brown oil. Column chromatographic purification [silica gel, hexane–AcOEt (2 : 1, v/v)] of this oil yielded **18a** (631 mg, 82% from **16a**) as a faintly yellowish oil, MS m/z: 365 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1660 (C=C), 1605 (lactam CO) (shifted to 1648 and 1576 cm^{-1}, respectively, on addition of an equimolar amount of $CHCl_2CO_2H$)[13]; ^1H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7Hz, $CH_2\underline{Me}$), 1.15—1.5 (2H, m, $C\underline{H}_2Me$), 2.20 (1H, br, $H_{(5)}$), 3.86 (3H, s, OMe), 5.14 (2H, s, $OC\underline{H}_2Ph$), 5.85 (1H, dd, J=10, 2 Hz, $H_{(3)}$), 6.40 (1H, dd, J=10, 3.5 Hz, $H_{(4)}$), 6.80 [3H, s (br), aromatic protons], 7.2—7.5 (5H, m, Ph).

1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-5,6-dihydro-2(1*H*)-pyridinone (18b)——Dehydrosulfenylation of **16b** through the sulfoxide **17b** was effected as described above for **18a**, and **18b** was obtained in 67% overall yield as a yellow oil, MS m/z: 365 (M^+); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1660 (C=C), 1603 (lactam CO) (shifted to 1648 and 1576 cm^{-1}, respectively, on addition of an equimolar amount of $CHCl_2CO_2H$)[13]; ^1H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=7Hz, $CH_2\underline{Me}$), 1.2—1.55 (2H, m, $C\underline{H}_2Me$), 2.25 (1H, br, $H_{(5)}$), 3.88 (3H, s, OMe), 5.12 (2H, s, $OC\underline{H}_2Ph$), 5.87 (1H, dd, J=9.5, 2 Hz, $H_{(3)}$), 6.42 (1H, dd, J=9.5, 3.5 Hz, $H_{(4)}$), 6.68 (1H, dd, J=8, 1.5 Hz, $H_{(6')}$), 6.79 (1H, d, J=1.5 Hz, $H_{(2')}$), 6.82 (1H, d, J=8 Hz, $H_{(5')}$), 7.2—7.5 (5H, m, Ph).[29]

1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidinemalonic Acid (19a)——A solution of **18a** (548 mg, 1.5 mmol) in abs. EtOH (5 ml) was added dropwise over 30 min in an atmosphere of N_2 to a stirred solution of diethyl malonate (481 mg, 3 mmol) in a mixture of Na (53 mg, 2.3 mg.-atom) and abs. EtOH (5 ml). The resulting solution was heated at 70 °C for 2 h and then under reflux for 6 h. After cooling, AcOH (0.3 ml) was added, and the mixture was concentrated *in vacuo* to leave an oil. The oil was partitioned by extraction with a mixture of H_2O and benzene, and the benzene extracts were washed successively with 5% aqueous Na_2CO_3 and saturated aqueous NaCl, dried, and concentrated to give a yellow oil (871 mg). The excess of diethyl malonate was removed from this oil by vacuum distillation [120 °C (bath temp.) at 4 mmHg, 2 h], and the oily, yellow residue (678 mg) was chromatographed on a silica gel column using hexane–AcOEt (1 : 1, v/v) as the eluent. Later fractions gave a yellow oil (583 mg), which was shown to be a mixture of two components on thin-layer chromatographic (TLC) analysis. The total amount of the oil was dissolved in EtOH (5 ml), and the solution was heated, after addition of 1 N aqueous NaOH (2 ml), at 50 °C for 4 h. The reaction mixture was concentrated *in vacuo*, and the residue was partitioned by extraction with a mixture

of H_2O (20 ml) and benzene. The aqueous extracts were acidified with 10% aqueous HCl to pH 2 and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with saturated aqueous NaCl, dried, and concentrated to leave crude **19a** (357 mg, 51% from **18a**) as a faintly brownish foam, presumed to be a mixture of the *trans* and *cis* isomers. This sample was directly used in the next decarboxylation step without further purification.

1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidinemalonic Acid (19b)——The Michael reaction of **18b** (1.47 g, 4 mmol) with diethyl malonate was effected as described above for **19a**, and alkaline hydrolysis of the resulting crude adduct (1.84 g) was carried out in EtOH (10 ml) containing 2 N aqueous NaOH (4 ml) at 50 °C for 20 h. The malonic acid **19b**, obtained as a faintly brownish foam (1.16 g, 61% from **18b**) (presumed to be a mixture of the *trans* and *cis* isomers), was directly used in the next decarboxylation step without further purification.

(±)-*trans*-1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidineacetic Acid (20a)——A solution of **19a** (357 mg, 0.76 mmol) in 60% aqueous AcOH (10 ml) was heated under reflux for 6 h. The reaction mixture was concentrated *in vacuo* to leave an oil, which was dissolved in benzene (20 ml). The benzene solution was washed with H_2O, dried, and concentrated to give a faintly yellow oil (319 mg). The ^{13}C-NMR spectroscopic analysis[32] of this oil suggested that it was an 83 : 17 mixture of the 4,5-*trans* isomer [^{13}C-NMR (Me_2SO-d_6) δ: 10.7 (CCH$_2$<u>Me</u>), 23.1 (C<u>C</u>H$_2$Me)] and the 4,5-*cis* isomer [δ: 11.6 (CCH$_2$<u>Me</u>), 20.4 (C<u>C</u>H$_2$Me)]. Crystallization of the mixture from hexane–$CHCl_3$, recrystallization of the resulting crystals from the same solvent, and drying over P_2O_5 at 2 mmHg and room temperature for 19 h gave the *trans* isomer **20a**·$CHCl_3$ (245 mg, 59%) as colorless needles, mp 117.5—119 °C; MS *m/z*: 425 (M^+); IR ν_{max}^{Nujol} cm^{-1}: 1719 (CO_2H), 1605 (lactam CO); ^1H-NMR (Me_2SO-d_6) δ: 0.77 (3H, t, J=6.5 Hz, CCH$_2$<u>Me</u>), 3.73 (3H, s, OMe), 5.05 (2H, s, OC<u>H</u>$_2$Ph), 6.72 (1H, dd, J=8.5, 1.5 Hz, $H_{(6')}$), 6.89 (1H, d, J=8.5 Hz, $H_{(5')}$), 6.93 (1H, d, J=1.5 Hz, $H_{(2')}$), 7.2—7.55 (5H, m, Ph), 8.31 (1H, s, $CHCl_3$), 12.18 (1H, br, CO_2H).[29] *Anal.* Calcd for $C_{25}H_{31}NO_5$·$CHCl_3$: C, 57.31; H, 5.92; N, 2.57. Found: C, 56.95; H, 5.92; N, 2.42. This sample showed a positive reaction in the Beilstein test for halogen, supporting its crystallization in the embrace of $CHCl_3$.

(±)-*trans*-1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidineacetic Acid (20b)——A solution of **19b** (939 mg, 2 mmol) in 60% aqueous AcOH (25 ml) was heated under reflux for 6 h. The reaction mixture was worked up as described above for **20a**, giving crude **20b** (810 mg) as a pale brown solid. On the basis of the results of ^{13}C-NMR spectroscopic analysis,[32] this solid was estimated to be a 90 : 10 mixture of the 4,5-*trans* isomer [^{13}C-NMR ($CDCl_3$) δ: 10.7 (CCH$_2$<u>Me</u>), 23.1 (C<u>C</u>H$_2$Me)] and the 4,5-*cis* isomer [δ: 11.9 (CCH$_2$<u>Me</u>), 20.8 (C<u>C</u>H$_2$Me)]. Recrystallizations of the solid first from hexane–AcOEt (1 : 1, v/v) and then from AcOEt afforded the *trans* isomer **20b** (554 mg, 65%) as colorless needles, mp 126—126.5 °C; IR ν_{max}^{Nujol} cm^{-1}: 1719 (CO_2H), 1600 (lactam CO); ^1H-NMR ($CDCl_3$) δ: 0.79 (3H, t, J=6.5 Hz, CCH$_2$<u>Me</u>), 3.88 (3H, s, OMe), 5.11 (2H, s, OC<u>H</u>$_2$Ph), 6.66 (1H, dd, J=8, 2 Hz, $H_{(6')}$), 6.77 (1H, d, J= 2 Hz, $H_{(2')}$), 6.82 (1H, d, J=8 Hz, $H_{(5')}$), 7.2—7.55 (5H, m, Ph), 10.84 (1H, br, CO_2H).[29] *Anal.* Calcd for $C_{25}H_{31}NO_5$: C, 70.57; H, 7.34; N, 3.29. Found: C, 70.34; H, 7.40; N, 3.26.

(±)-*trans*-1-(3-Benzyloxy-4-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidineacetic Acid Ethyl Ester (21a)——A solution of **20a** (136 mg, 0.25 mmol) in 10% (w/w) ethanolic HCl (3 ml) was stirred at room temperature for 24 h. The reaction mixture was concentrated *in vacuo*, and the residue was partitioned by extraction with a mixture of benzene and H_2O. The benzene extracts were washed successively with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, dried, and concentrated to leave **21a** (103 mg, 91%) as a colorless oil. This sample was identical [by comparison of the IR ($CHCl_3$) and ^1H-NMR ($CDCl_3$) spectra and TLC mobility] with authentic **21a**.[16]

(±)-*trans*-1-(4-Benzyloxy-3-methoxyphenethyl)-5-ethyl-2-oxo-4-piperidineacetic Acid Ethyl Ester (21b)——Esterification of **20b** was achieved as described above for **21a**, and **21b** was obtained in 89% yield as a colorless oil. This specimen was identical [by comparison of the IR ($CHCl_3$) and ^1H-NMR ($CDCl_3$) spectra and TLC mobility] with authentic **21b**.[16]

3-Benzyloxy-4-methoxyphenylacetic Acid (24a)——The following procedure is a modification of that reported in the literature.[22] A mixture of **22a**[22] (40.0 g, 156 mmol), sulfur (10.0 g, 312 mg.-atom), and morpholine (27.2 g, 312 mmol) was heated at 80 °C for 1 h and then under reflux for 5 h. The reaction mixture was diluted with benzene (400 ml), and the resulting benzene solution was washed successively with 10% aqueous HCl, 10% aqueous Na_2CO_3, and saturated aqueous NaCl, dried, and concentrated to leave crude thiomorpholide **23a** as a yellowish brown solid (50.0 g). A mixture of the total amount of the solid and 50% aqueous KOH (170 ml) in EtOH (500 ml) was heated under reflux for 9 h. After cooling, the reaction mixture was concentrated *in vacuo*, and the residue was partitioned by extraction with a mixture of H_2O (500 ml) and benzene. The aqueous extracts were made acid to Congo red with conc. aqueous HCl and extracted with $CHCl_3$. The $CHCl_3$ extracts were washed successively with H_2O and saturated aqueous NaCl, dried, and concentrated to leave a brown solid. Recrystallization of the solid from benzene gave **24a** (25.0 g, 59% from **22a**) as pale brownish needles, mp 123.5—125.5 °C (lit.[22] mp 122 °C); IR ν_{max}^{Nujol} cm^{-1}: 1705 (CO_2H); ^1H-NMR ($CDCl_3$) δ: 3.53 (2H, s, ArC<u>H</u>$_2$), 3.86 (3H, s, OMe), 5.12 (2H, s, OC<u>H</u>$_2$Ph), 6.84 [3H, s (br), aromatic protons], 7.2—7.6 (5H, m, Ph), 9.4 (1H, br, CO_2H).

4-Benzyloxy-3-methoxyphenylacetic Acid (24b)——A mixture of **22b**[30,33] (25.7 g, 100 mmol), sulfur (4.81 g, 150 mg.-atom), and morpholine (13.1 g, 150 mmol) was heated at 80 °C for 1 h and then under reflux for 5 h. The reaction mixture was worked up as described above for **24a**, affording the crude thiomorpholide **23b** as a brown oil (35.0 g). The oil was hydrolyzed with 50% aqueous KOH (100 ml) in boiling EtOH (300 ml) for 5 h. Work-up of the resulting ethanolic solution in a manner similar to that described above for **24a** yielded a yellowish brown solid

(20.9 g). Recrystallization of the solid from EtOH–H_2O (1 : 2, v/v) gave **24b** (16.4 g, 60% from **22b**) as faintly yellowish needles, mp 117—118 °C (lit.[34] mp 114—115 °C); IR ν_{max}^{Nujol} cm^{-1}: 1707 (CO_2H); ^{1}H-NMR ($CDCl_3$) δ: 3.56 (2H, s, ArCH_2), 3.87 (3H, s, OMe), 5.13 (2H, s, OCH_2Ph), 6.73 (1H, dd, J=8, 2 Hz, $H_{(6)}$), 6.81 (1H, d, J=2 Hz, $H_{(2)}$), 6.85 (1H, d, J=8 Hz, $H_{(5)}$), 7.2—7.5 (5H, m, Ph), 9.2 (1H, br, CO_2H).

3-Benzyloxy-4-methoxyphenylacetic Acid Ethyl Ester (25a)——A mixture of **24a** (545 mg, 2 mmol) and 10% (w/w) ethanolic HCl (3 ml) in abs. EtOH (3 ml) was stirred at 30 °C for 17 h. The reaction mixture was concentrated *in vacuo*, and the residue was dissolved in benzene (70 ml). The benzene solution was washed successively with H_2O, 5% aqueous NaOH, and saturated aqueous NaCl, dried, and concentrated to leave **25a** (565 mg, 94%) as a colorless solid, mp 69—70 °C. Recrystallization from hexane yielded a pure sample as colorless needles, mp 69—70 °C (lit.[35] mp 66—67 °C); IR ν_{max}^{Nujol} cm^{-1}: 1731 (ester CO); ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, J=7 Hz, OCH_2Me), 3.50 (2H, s, ArCH_2), 3.86 (3H, s, OMe), 4.11 (2H, q, J=7 Hz, OCH_2Me), 5.14 (2H, s, OCH_2Ph), 6.75—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph).

4-Benzyloxy-3-methoxyphenylacetic Acid Ethyl Ester (25b)——A mixture of **24b** (15.0 g, 55 mmol) and 10% (w/w) ethanolic HCl (55 ml) in abs. EtOH (100 ml) was stirred at room temperature for 24 h. The reaction mixture was worked up as described above for **25a**, giving **25b** (15.6 g, 94%) as a brown solid, mp 43.5—44.5 °C. Recrystallization from ether–hexane (1 : 4, v/v) furnished an analytical sample as colorless needles, mp 44.5—45.5 °C; MS *m/z*: 300 (M^+); IR ν_{max}^{Nujol} cm^{-1}: 1723 (ester CO); ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7 Hz, OCH_2Me), 3.53 (2H, s, ArCH_2), 3.88 (3H, s, OMe), 4.15 (2H, q, J=7 Hz, OCH_2Me), 5.13 (2H, s, OCH_2Ph), 6.6—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{18}H_{20}O_4$: C, 71.98; H, 6.71. Found: C, 71.95; H, 6.76.

3-Benzyloxy-4-methoxyphenethyl Alcohol (26a)——To a stirred, chilled (−5 °C) suspension of $LiAlH_4$ (2.24 g, 59 mmol) in dry ether (200 ml) was added dropwise a solution of **25a** (17.7 g, 59 mmol) in dry ether (800 ml) at such a rate that the inner temperature was kept below 0 °C. After the mixture had been stirred at room temperature for 2 h, H_2O (2.5 ml), 15% aqueous NaOH (2.5 ml), and H_2O (30 ml) were added dropwise in that order under ice-cooling and stirring. The insoluble material that resulted was removed by filtration, and the filtrate was dried and concentrated to leave **26a** (14.8 g, 97%) as a colorless solid, mp 80—80.5 °C. Recrystallization from hexane–AcOEt (3 : 1, v/v) produced an analytical sample as colorless needles, mp 80—80.5 °C; IR ν_{max}^{Nujol} cm^{-1}: 3360, 3300 (OH); ^{1}H-NMR ($CDCl_3$) δ: 1.44 (1H, s, OH), 2.74 (2H, t, J=6.5 Hz, ArCH_2), 3.76 (2H, t, J=6.5 Hz, CH_2OH), 3.86 (3H, s, OMe), 5.14 (2H, s, OCH_2Ph), 6.6—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{16}H_{18}O_3$: C, 74.40; H, 7.02. Found: C, 74.49; H, 7.04.

4-Benzyloxy-3-methoxyphenethyl Alcohol (26b)——This was obtained in 96% yield from **25b** by $LiAlH_4$ reduction similar to that described above for **26a** and recrystallized from hexane–AcOEt (4 : 1, v/v) to afford colorless needles, mp 69—70 °C (lit. mp 70—70.5 °C[36a]; mp 72 °C[36b]); IR ν_{max}^{Nujol} cm^{-1}: 3590 (OH); ^{1}H-NMR ($CDCl_3$) δ: 1.55 (1H, s, OH), 2.80 (2H, t, J=6.5 Hz, ArCH_2), 3.83 (2H, t, J=6.5 Hz, CH_2OH), 3.88 (3H, s, OMe), 5.13 (2H, s, OCH_2Ph), 6.69 (1H, dd, J=8, 2 Hz, $H_{(6)}$), 6.78 (1H, d, J=2 Hz, $H_{(2)}$), 6.84 (1H, d, J=8 Hz, $H_{(5)}$), 7.2—7.5 (5H, m, Ph).

3-Benzyloxy-4-methoxyphenethyl Bromide (27a)——Triphenylphosphine (14.4 g, 55 mmol) was added portionwise to a stirred solution of **26a** (12.9 g, 50 mmol) in benzene (100 ml). *N*-Bromosuccinimide (9.34 g, 52.5 mmol) was then added portionwise at such a rate that the inner temperature did not exceed 10 °C. After having been stirred at room temperature for 5 h, the reaction mixture was filtered. The filtrate was washed successively with 5% aqueous $Na_2S_2O_3$, 5% aqueous NaOH, and saturated aqueous NaCl, dried, and concentrated. The residue was chromatographed on a 150-g silical gel column ($CHCl_3$) to give a colorless solid (15.4 g). The solid was recrystallized from hexane–AcOEt to yield **27a** (12.5 g) as colorless needles, mp 90.5—91.5 °C. Concentration of the mother liquor of this recrystallization and column chromatography of the residue [silica gel, benzene–hexane (1 : 1, v/v)] provided a second crop (2.42 g) of **27a**. The total yield of **27a** was 14.9 g (93%). Further recrystallization from hexane–AcOEt (3 : 1, v/v) gave an analytical sample as colorless needles, mp 91—91.5 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.9—3.15 (2H, m, ArCH_2), 3.35—3.55 (2H, m, CH_2Br), 3.87 (3H, s, OMe), 5.14 (2H, s, OCH_2Ph), 6.6—6.9 (3H, m, aromatic protons), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{16}H_{17}BrO_2$: C, 59.83; H, 5.33. Found: C, 60.03; H, 5.35.

4-Benzyloxy-3-methoxyphenethyl Bromide (27b)——The alcohol **26b** (12.4 g, 48 mmol) was brominated in a manner similar to that described above for **27a**, and the crude solid that obtained was purified by column chromatography [silica gel, benzene–hexane (1 : 1, v/v)] to afford **27b** (14.2 g, 92%) as a colorless solid, mp 45.5—47 °C. Recrystallization from hexane gave an analytical sample as colorless needles, mp 45.5—47 °C; ^{1}H-NMR ($CDCl_3$) δ: 3.09 (2H, t, J=7.5 Hz, ArCH_2), 3.53 (2H, t, J=7.5 Hz, CH_2Br), 3.88 (3H, s, OMe), 5.13 (2H, s, OCH_2Ph), 6.67 (1H, dd, J=8, 2 Hz, $H_{(6)}$), 6.75 (1H, d, J=2 Hz, $H_{(2)}$), 6.83 (1H, d, J=8 Hz, $H_{(5)}$), 7.2—7.5 (5H, m, Ph). *Anal.* Calcd for $C_{16}H_{17}BrO_2$: C, 59.83; H, 5.33. Found: C, 59.71; H, 5.26.

1-(3-Benzyloxy-4-methoxyphenethyl)-3-(1,1-ethylenedioxyethyl)pyridinium Bromide (29a)——A mixture of **5**[27c] (3.80 g, 23 mmol) and **27a** (8.13 g, 25.3 mmol) in dry benzene (21 ml) was heated under reflux for 72 h to form two layers. The lower, oily layer was separated from the upper benzene layer by decantation and dissolved in H_2O (18 ml). The aqueous solution was extracted with benzene, and the benzene extracts were combined with the benzene layer described above, dried, and concentrated *in vacuo*. The residue was dissolved in dry benzene (5 ml), and the benzene solution was heated under reflux for 24 h. The reaction mixture was worked up as in the above first cycle, and the

aqueous extracts were combined with those of the first cycle and concentrated *in vacuo* to leave crude **29a** (10.8 g) as a yellow oil. This oil was directly used in the next oxidation step without further purification.

1-(4-Benzyloxy-3-methoxyphenethyl)-3-(1,1-ethylenedioxyethyl)pyridinium Bromide (29b)——A mixture of **5**[27c] (4.96 g, 30 mmol) and **27b** (10.1 g, 31.4 mmol) in dry benzene (30 ml) was heated under reflux for 72 h. Extraction of the reaction mixture with H_2O (75 ml) and concentration of the aqueous extracts under reduced pressure left **29b** (13.2 g) as a yellow oil. The oil was directly used in the next oxidation step without further purification.

1-(3-Benzyloxy-4-methoxyphenethyl)-3-(1,1-ethylenedithioethyl)pyridinium Bromide (30a)——A mixture of **28**[27c] (1.38 g, 7 mmol) and **27a** (2.47 g, 7.7 mmol) in dry benzene (7 ml) was heated under reflux for 72 h. After cooling, the reaction mixture was extracted with H_2O (90 ml), and the aqueous extracts were concentrated *in vacuo* to leave crude **30a** (3.60 g) as a yellow oil. The oil was directly used in the next oxidation step without further purification.

1-(3-Benzyloxy-4-methoxyphenethyl)-5-(1,1-ethylenedioxyethyl)-2(1*H*)-pyridinone (31a)——According to the previously reported standard procedure,[9a,24] a solution of the total amount of the crude **29a** described above in H_2O (33 ml) was oxidized with a solution of $K_3Fe(CN)_6$ (43.8 g, 133 mmol) in H_2O (130 ml) and a solution of KOH (19.9 g, 355 mmol) in H_2O (30 ml) at 32 °C for 5 h in the presence of benzene (74 ml). After cooling, the benzene layer was separated from the aqueous layer, which was further extracted with benzene. The benzene layer and the benzene extracts were combined, dried, and concentrated to leave a dark, reddish brown oil (8.71 g). Purification of the oil by column chromatography [alumina, hexane–AcOEt (1 : 1, v/v)] furnished **31a** (8.27 g, 85% yield from **5**) as a yellowish orange oil, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1666 (pyridone CO); UV λ_{max}^{EtOH} 231.5 nm (ε 15400), 287 (5350), 307.5 (5350); ^{1}H-NMR ($CDCl_3$) δ: 1.46 (3H, s, CMe), 2.96 (2H, t, J=7 Hz, ArC$\underline{H}_2$), 3.4—4.0 (4H, m, OCH$_2$'s), 3.82 (3H, s, OMe), 4.05 (2H, t, J=7 Hz, NCH$_2$), 5.03 (2H, s, OC$\underline{H}_2$Ph), 6.55 (1H, d, J=9.5 Hz, $H_{(3)}$), 6.55—6.85 (3H, m, aromatic protons), 6.81 (1H, d, J=2.5 Hz, $H_{(6)}$), 7.2—7.5 (5H, m, Ph), 7.34 (1H, dd, J=9.5, 2.5 Hz, $H_{(4)}$).

1-(4-Benzyloxy-3-methoxyphenethyl)-5-(1,1-ethylenedioxyethyl)-2(1*H*)-pyridinone (31b)——The total amount of the above crude **29b** was oxidized in the same manner as described above for **31a**, giving **31b** (86% yield from **5**) as a reddish brown oil, MS *m/z*: 421 (M$^+$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1666 (pyridone CO); UV λ_{max}^{EtOH} 232 nm (ε 16500), 287 (5300), 308 (5350); ^{1}H-NMR ($CDCl_3$) δ: 1.45 (3H, s, CMe), 3.00 (2H, t, J=6.5 Hz, ArC$\underline{H}_2$), 3.3—3.9 (4H, m, OCH$_2$'s), 3.78 (3H, s, OMe), 4.10 (2H, t, J=6.5 Hz, NCH$_2$), 5.09 (2H, s, OC$\underline{H}_2$Ph), 6.5—6.9 (3H, m, aromatic protons), 6.56 (1H, d, J=9.5 Hz, $H_{(3)}$), 6.86 (1H, d, J=2.5 Hz, $H_{(6)}$), 7.2—7.5 (5H, m, Ph), 7.34 (1H, dd, J=9.5, 2.5 Hz, $H_{(4)}$).

1-(3-Benzyloxy-4-methoxyphenethyl)-5-(1,1-ethylenedithioethyl)-2(1*H*)-pyridinone (32a)——The total amount of the above crude **30a** was oxidized in the same way as described above for **31a**. The crude product was purified by column chromatography [silica gel, hexane–AcOEt (1 : 1, v/v)] to give **32a** (67% yield from **28**) as a pale brown solid. Recrystallization from hexane–AcOEt (1 : 1, v/v) and drying over P_2O_5 at 2 mmHg and room temperature for 24 h yielded an analytical sample as faintly brownish pillars, mp 92.5—98 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1662 (pyridone CO); UV λ_{max}^{EtOH} 234 nm (ε 19200), 283.5 (4600), 313.5 (4750); ^{1}H-NMR ($CDCl_3$) δ: 1.84 (3H, s, CMe), 2.96 (2H, t, J=6.5 Hz, ArC$\underline{H}_2$), 3.0—3.5 (4H, m, SCH$_2$'s), 3.85 (3H, s, OMe), 4.06 (2H, t, J=6.5 Hz, NCH$_2$), 5.04 (2H, s, OC$\underline{H}_2$Ph), 6.5—6.85 (3H, m, aromatic protons), 6.59 (1H, d, J=9.5 Hz, $H_{(3)}$), 6.96 (1H, d, J=3 Hz, $H_{(6)}$), 7.2—7.5 (5H, m, Ph), 7.61 (1H, dd, J=9.5, 3 Hz, $H_{(4)}$). *Anal.* Calcd for $C_{25}H_{27}NO_3S_2 \cdot 1/2H_2O$: C, 64.91; H, 6.10; N, 3.03. Found: C, 64.65; H, 6.13; N, 2.80.

5-(1,1-Ethylenedioxyethyl)-1-(3-hydroxy-4-methoxyphenethyl)-2-piperidone (33)——A solution of **31a** (8.01 g, 19 mmol) in EtOH (150 ml) was hydrogenated over Raney Ni W-2 catalyst (10 ml) at atmospheric pressure and room temperature for 13 h. Removal of the catalyst by filtration and concentration of the filtrate under reduced pressure afforded a colorless oil, which was dissolved in $CHCl_3$ (100 ml). The $CHCl_3$ solution was dried and concentrated to leave **33** (6.20 g, 97%) as a colorless oil. The oil was crystallized from hexane–AcOEt (1 : 1, v/v) to give an analytical sample as colorless needles, mp 102.5—103.5 °C; MS *m/z*: 335 (M$^+$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1627 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, s, CMe), 3.8—4.0 (4H, m, OCH$_2$'s), 3.86 (3H, s, OMe), 5.9 (1H, br, OH), 6.6—6.85 (3H, m, aromatic protons). *Anal.* Calcd for $C_{18}H_{25}NO_5$: C, 64.46; H, 7.51; N, 4.18. Found: C, 64.34; H, 7.69; N, 4.16.

5-(1,1-Ethylenedioxyethyl)-1-(4-hydroxy-3-methoxyphenethyl)-2-piperidone (34)——A solution of **31b** (10.1 g, 24 mmol) in EtOH (100 ml) was hydrogenated over Raney Ni W-2 catalyst (15 ml) at atmospheric pressure and room temperature for 22 h. The reaction mixture was worked up as described above for **33**, giving **34** (7.94 g, 99%) as a faintly yellowish oil, which was shown to be homogeneous on TLC analysis. Purification of the oil by column chromatography (silica gel, AcOEt) provided a colorless oil, MS *m/z*: 335 (M$^+$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3560 (OH), 1627 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, s, CMe), 3.8—4.0 (4H, m, OCH$_2$'s), 3.88 (3H, s, OMe), 4.5 (1H, br, OH), 6.6—6.9 (3H, m, aromatic protons).

Deketalization of 33——A mixture of **33** (335 mg, 1 mmol) and 10% aqueous HCl (1 ml) in EtOH (3 ml) was stirred at 50 °C for 2 h. The reaction mixture was concentrated *in vacuo*, and the oily residue was extracted with $CHCl_3$ after addition of H_2O (5 ml) and subsequent neutralization with $NaHCO_3$. The $CHCl_3$ extracts were dried and concentrated to leave **11** (280 mg, 96%) as a faintly brown solid, mp 92—93 °C. Recrystallization from hexane–AcOEt produced colorless needles, mp 99—100 °C, which were identical [by mixture melting point test and comparison of the IR (Nujol) spectrum and TLC mobility] with authentic **11** prepared by the foregoing alternative route.

Deketalization of 34——A mixture of **34** (671 mg, 2 mmol) and 10% aqueous HCl (2 ml) in EtOH (6 ml) was

stirred at 50 °C for 2 h. The reaction mixture was worked up as in the case of the above deketalization of **33**, giving **12** (571 mg, 98%) as a colorless solid, mp 96—98 °C. Recrystallization from benzene–hexane (3:1, v/v) furnished colorless prisms, mp 116—116.5 °C, which were identical [by mixture melting point test and comparison of the IR (Nujol) spectrum and TLC mobility] with authentic **12** obtained by the foregoing alternative route.

Reductive Desulfurization of 32a——A mixture of **32a**·$1/2H_2O$ (278 mg, 0.6 mmol), 70% aqueous EtOH (20 ml), and Raney Ni[37] (3 ml) was heated under reflux for 1 h. The catalyst was filtered off and washed with EtOH. The filtrate and ethanolic washings were combined and concentrated *in vacuo* to leave a colorless oil, which was dissolved in $CHCl_3$ (50 ml). The $CHCl_3$ solution was washed with saturated aqueous NaCl, dried, and concentrated. The resulting colorless oily residue (150 mg) was chromatographed on a 57-g silica gel column using $CHCl_3$–MeOH (50:1, v/v) as the eluent. Earlier fractions yielded **13** (110 mg, 66%) as a faintly yellow solid, mp 117.5—118.5 °C, which was identical [by mixture melting point test and comparison of the IR (Nujol) spectrum and TLC mobility] with authentic **13** synthesized by the foregoing alternative route.

Later fractions of the chromatography gave, on concentration, the demethoxylated product **36** (30 mg, 20%) as a colorless solid, mp 120—121.5 °C. Recrystallization from hexane–AcOEt afforded a pure sample as colorless needles, mp 124—125 °C, which were identical [by mixture melting point test and comparison of the IR (Nujol) and ^{1}H-NMR ($CDCl_3$) spectra and TLC mobility] with authentic **36** described below.

5-Ethyl-1-(3-hydroxyphenethyl)-2-piperidone (36)——A solution of **35**[19,26] (1.06 g, 3 mmol) in EtOH (30 ml) containing 70% aqueous $HClO_4$ (0.5 ml) was hydrogenated over 10% Pd–C (800 mg) at 3.7—4 atm and room temperature for 10 h. The catalyst was removed by filtration and washed with EtOH (40 ml). The filtrate and washings were combined and concentrated *in vacuo*, and the residue was partitioned by extraction with a mixture of H_2O and $CHCl_3$. The $CHCl_3$ extracts were washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, dried, and concentrated to leave a slightly brown solid (694 mg, 94%), mp 113.5—119 °C. Recrystallization from hexane–AcOEt (1:1, v/v) gave an analytical sample of **36** as colorless needles, mp 124—125 °C; IR ν_{max}^{Nujol} cm^{-1}: 3160, 3100 (OH), 1615 (lactam CO); ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7 Hz, $CCH_2\underline{Me}$), 3.4—3.7 (2H, m, $ArCH_2C\underline{H}_2N$), 6.6—6.8 (2H, m, $H_{(4')}$ and $H_{(6')}$), 6.88 (1H, dull t, J=2 Hz, $H_{(2')}$), 7.15 (1H, t, J=7.5 Hz, $H_{(5')}$).[29] *Anal*. Calcd for $C_{15}H_{21}NO_2$: C, 72.84; H, 8.56; N, 5.66. Found: C, 72.62; H, 8.73; N, 5.62.

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Photochemistry of Conjugated Nitrogen–Thiocarbonyl Systems. V.[1a)] Two-Fold Photoaddition of Vinyl Ethers to Aza-Aromatic Thiones[1b)]

EISUKE SATO, YOSHIYA IKEDA and YUICHI KANAOKA*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan

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Irradiation of 2-thiopyridone (**1**), quinoline-2-thione (**10**), isoquinoline-1-thione (**18h**) and phthalazine-1-thione (**18i**) with vinyl ethers (**7**) gave two-fold addition products, by addition of the olefins to the C=S bond.

Keywords——photoaddition; vinyl ether; olefin; two-fold addition; 2-thiopyridone; quinoline-2-thione; isoquinoline-1-thione; phthalazine-1-thione

A unique photoreaction of the conjugated nitrogen–thione system **1** is "two-fold addition" of certain olefins. For example, irradiation of 2-thiopyridone,[2)] 2-thiopyrimidone[2)] and quinoline-2-thione,[1a)] in the presence of excess isobutene **2**, gave the aza-aromatics substituted at the thiocarbonyl carbon with a thioether side chain which incorporates two molecules of isobutene. This novel addition may be explained according to the mechanism depicted in Chart 1,[2)] which takes into account the intermediacy of the thietane **3** and its subsequent photolysis to a biradical **4**. This thiyl biradical would be reactive and would immediately take up another molecule of isobutene at its terminal thiyl radical, leading ultimately to the 2-thioether derivative (**5**→**6**).

{ : benzene or H
X: CH or N

Chart 1

Since a thiyl radical is electrophilic,[3)] olefins with electron-donating substituents may be more susceptible to the reaction with such thiyl radicals. Olefins with a vinyl group were selected as suitable candidates. Thus photoaddition of various vinyl ethers (**7**) to 2-thiopyridone and other related bicyclic thiocarbonyls was examined in the expectation of an efficient two-fold reaction, and the results are reported in the present paper.

Irradiation of 2-thiopyridone (**1**) in acetonitrile in the presence of excess vinyl ethers (**7**), with a 500 W high-pressure mercury lamp through a Pyrex filter for 3—5 h under an argon atmosphere, afforded the expected two-fold addition products **8** with a small amount of disulfide **9** (Chart 2 and Table I).

In the reaction of vinyl ethers **7** with isobutene, the two-fold addition predominates over the formation of the disulfide **9**, suggesting that addition of the thiyl radical to an olefin at the second step is enhanced by the use of electron-rich olefins such as **7**.

a: R=Me b: R=Et c: R=iso-Pr d: R=*n*-Bu e: R=iso-Bu

Chart 2

TABLE I. Yields of Photoproducts

Thione (mmol)	Olefin (7) (mmol)	Time (h)	Product, % yield					
			8	9	11	12	19	20
1 (1)	**7a** (10)	3	19	3				
1 (1)	**7b** (10)	3	19	6				
1 (1)	**7c** (10)	4	46	3				
1 (1)	**7d** (10)	5	59	7				
1 (1)	**7e** (10)	5	54	10				
10f (1)	**7b** (10)	3			17	12		
10f (1)	**7b** (30)	1			23	13		
10g (1)	**7b** (10)	3			14	11		
10f (1)	**7c** (10)	3			15	25		
10f (1)	**7d** (10)	4			24	8		
10f (1)	**7e** (10)	4			14	26		
18h (1)	**7b** (10)	3					13	7
18i (1)	**7b** (10)	3					23	0

f: X=H g: X=CH_3

Chart 3

Photoaddition of the olefins (7) to bicyclic thiones **10** was then examined, since quinoline-2-thiones show similar photochemical behavior to that of 2-thiopyridone.[1a] On irradiation of quinoline-2-thione in the presence of the olefins 7, two kinds of two-fold adducts (**11** and **12**) were obtained. The former adducts (**11**) are the same kind of addition products as those obtained in the case of 2-thiopyridone, but the latter adducts (**12**) are a new type, resulting from elimination of one of the ether moieties (Chart 3).

As shown in Chart 4, the formation of **12** can be explained by intramolecular hydrogen abstraction at the carbon adjacent to the ether oxygen by a radical at the side chain of the biradical **15**, followed by elimination of a ketone or aldehyde. In fact, for example, acetaldehyde, the eliminated product, was identified as its 2,4-dinitrophenylhydrazone in the reaction of **10f** with **7b**. The better yield of **12fc** than of **12fb** (Table I) can be explained by the fact that the rates of hydrogen abstraction from aliphatic hydrocarbons increase in the order primary < secondary < tertiary.[4] These results are consistent with the postulated intra-

Chart 4

Chart 5

molecular hydrogen abstraction mechanism among side chains shown in Chart 4. The radical at the quinoline ring **15** is considerably stabilized by the bicyclic conjugation, compared with the similar type of radical formed in the reaction of 2-thiopyridone and vinyl ether, and this would be one of the reasons why the above intramolecular hydrogen abstraction in the side chain occurs to give the new products (**12**).

This two-fold addition was further examined with other bicyclic heterocycles such as isoquinoline-1-thione (**18h**) and phthalazine-1-thione (**18i**). Irradiation of **18h** with **7b** gave the expected two-fold addition product **19h** together with the disulfide **20**, and irradiation of **18i** in methanol with **7b** also afforded the two-fold adduct **19i** (Chart 5).

Recently, Omote *et al.*[5] have also reported that photoreactions of 2-thiopyridone and quinoline-2-thione with electron-deficient olefins gave β-mercaptoalkylated products. In addition to this kind of substitution initiated by Paterno–Büchi reaction, we have now established that aza-aromatic thione systems generally undergo the unique two-fold addition reaction. From the above results and the previous work[2] at least the following two conditions seem to be required for the occurrence of this "two-fold addition": (i) low steric hindrance in the vicinity of the thiyl radical of the intermediate biradical (**4**) generated by photolysis of thietane; (ii) a relatively electron-rich olefin, which is more susceptible to attack of the electrophilic thiyl radical. Vinyl ethers and isobutene apparently satisfy both conditions.

Experimental

Vacuum distillation was carried out using a Büchi Kugelrohr apparatus and boiling points are uncorrected. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM-FX 100 FT-NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were obtained with a JMS-D 300 mass spectrometer. The light source was a Type EHBW1 (Eiko-sha) 500 W high-pressure mercury lamp.

TABLE II. Spectral Data for Photoproducts

Product	MS m/z	^{1}H-NMR ($CDCl_3$) δ (ppm) J (Hz)
8a	227 (M^+) 196	2.71 (2H, t, J=7), 2.97 (2H, d, J=6), 3.34 (3H, s), 3.36 (3H, s), 3.52 (2H, t, J=7), 4.47 (1H, t, J=6), 7.12—7.27 (1H, m), 7.36—7.46 (1H, m), 7.64—7.79 (1H, m), 8.50—8.61 (1H, m)
9a	336 (M^+) 304	2.95 (4H, d, J=6), 3.34 (6H, s), 4.45 (2H, t, J=6), 7.10—7.50 (4H, m), 7.62—7.78 (2H, m), 8.50—8.59 (2H, m)
8b	255 (M^+) 210 209	1.19 (3H, t, J=6), 1.24 (3H, t, J=6), 2.73 (2H, t, J=6), 2.97 (2H, d, J=6), 3.39—3.64 (6H, m), 4.57 (1H, t, J=6), 7.13—7.27 (1H, m), 7.39—7.49 (1H, m), 7.63—7.80 (1H, m), 8.53—8.61 (1H, m)
9b	364 (M^+) 182	1.21 (6H, t, J=7), 3.09—3.19 (4H, m), 3.51 (4H, q, J=7), 4.66 (2H, dd, J=7, 5), 7.13—7.27 (2H, m), 7.40—7.48 (2H, m), 7.62—7.77 (2H, m), 8.53—8.59 (2H, m)
8c	283 (base, M^+)	1.12 (3H, d, J=6), 1.14 (6H, d, J=6), 1.24 (3H, d, J=6), 2.70 (2H, t, J=7), 2.92 (1H, d, J=7), 2.93 (1H, d, J=5), 3.45—3.73 (4H, m), 4.66 (1H, dd, J=7, 5), 7.11—7.25 (1H, m), 7.44—7.52 (1H, m), 7.62—7.79 (1H, m), 8.51—8.58 (1H, m)
9c	392 (M^+) 196 (base)	1.10 (12H, d, J=6), 1.22 (6H, d, J=6), 1.23 (2H, d, J=6), 2.87—3.30 (4H, m), 3.63 (2H, sept, J=6), 4.71 and 4.78 (2H, t, J=7), 7.11—7.23 (2H, m), 7.43—7.51 (2H, m), 7.61—7.78 (2H, m), 8.50—8.57 (2H, m)
8d	311 (base, M^+)	0.90 (6H, t, J=7), 1.18—1.67 (8H, m), 2.72 (2H, t, J=7), 2.96 (2H, d, J=7), 3.36—3.63 (6H, m), 4.55 (1H, t, J=6), 7.13—7.27 (1H, m), 7.40—7.47 (1H, m), 7.64—7.78 (1H, m), 8.50—8.58 (1H, m)
9d	420 (M^+) 210	0.88 (6H, t, J=6), 1.19—1.71 (8H, m), 3.08—3.27 (4H, m), 3.44 (4H, t, J=6), 4.63 (2H, dd, J=7, 5), 7.11—7.26 (2H, m), 7.39—7.46 (2H, m), 7.61—7.78 (2H, m), 8.52—8.59 (2H, m)
8e	311 (M^+) 210	0.88 (6H, d, J=7), 0.92 (6H, d, J=7), 1.58—2.08 (2H, m), 2.73 (2H, t, J=7), 2.97 (2H, d, J=7), 3.18 (2H, d, J=7), 3.20 (2H, d, J=7), 3.56 (2H, t, J=7), 4.55 (1H, t, J=6), 7.13—7.27 (1H, m), 7.40—7.48 (1H, m), 7.63—7.80 (1H, m), 8.51—8.58 (1H, m)
9e	420 (M^+) 210 (base) 178	0.90 (12H, d, J=7), 1.89 (2H, sept, J=7), 3.09—3.23 (8H, m), 4.62 (2H, t, J=7), 7.13—7.25 (2H, m), 7.39—7.47 (2H, m), 7.62—7.79 (2H, m), 8.53—8.58 (2H, m)
11fb	305 (M^+) 260	1.17 (3H, t, J=7), 1.25 (3H, t, J=7), 2.77 (2H, t, J=7), 3.54 (2H, d, J=7), 3.43—3.64 (6H, m), 4.77 (1H, t, J=6), 7.52—7.78 (4H, m), 8.03—8.22 (2H, m)
12fb	261 (M^+) 216 215 188	1.19 (3H, t, J=7), 2.75 (2H, t, J=7), 2.98—3.33 (4H, m), 3.38—3.66 (4H, m), 7.35—7.80 (4H, m), 7.99—8.11 (2H, m)
11gb	319 (M^+) 274 246	1.18 (3H, t, J=7), 1.25 (3H, t, J=7), 2.73 (3H, d, J=1), 2.77 (2H, t, J=7), 3.02 (2H, d, J=6), 3.47 (2H, q, J=7), 3.53 (2H, q, J=7), 3.58 (2H, t, J=7), 4.71 (1H, t, J=6), 7.43—7.79 (3H, m), 7.95—8.12 (2H, m)
12gb	275 (M^+) 230 202	1.20 (3H, t, J=7), 2.68 (3H, d, J=1), 2.76 (2H, t, J=7), 2.96—3.32 (4H, m), 3.50 (2H, q, J=7), 3.57 (2H, t, J=7), 7.16 (1H, d, J=1), 7.42—7.76 (2H, m), 7.91—8.09 (2H, m)
11fc	333 (base, M^+)	1.13 (9H, d, J=6), 1.27 (3H, d, J=6), 2.75 (2H, t, J=7), 3.00 (2H, d, J=6), 3.56 (2H, t, J=7), 3.43—3.71 (2H, m), 4.86 (1H, t, J=6), 7.44—7.87 (4H, m), 8.02—8.22 (2H, m)
12fc	275 (M^+) 216 215 188 (base)	1.15 (6H, t, J=6), 2.74 (2H, t, J=7), 2.99—3.38 (4H, m), 3.59 (2H, t, J=7), 3.59 (1H, sep, J=6), 7.28—7.82 (4H, m), 8.00—8.13 (2H, m)
11fd	361 (base, M^+)	0.89 (3H, t, J=6), 0.89 (3H, t, J=7), 1.16—1.66 (8H, m), 2.77 (2H, t, J=7), 3.03 (2H, d, J=6), 3.34—3.64 (6H, m), 4.75 (1H, t, J=6), 7.46—7.87 (4H, m), 8.04—8.23 (2H, m)
12fd	289 (M^+) 216 215 188	0.90 (3H, t, J=7), 1.17—1.72 (4H, m), 2.75 (2H, t, J=7), 2.98—3.34 (4H, m), 3.42 (2H, t, J=7), 3.59 (2H, t, J=7), 7.28—7.84 (4H, m), 7.99—8.12 (2H, m)
11fe	361 (M^+) 288 260	0.87 (6H, d, J=7), 0.93 (6H, d, J=7), 1.10—1.64 (2H, m), 2.77 (2H, t, J=6), 3.05 (2H, d, J=6), 3.00—3.37 (4H, m), 3.56 (2H, t, J=6), 4.74 (1H, t, J=6), 7.45—7.84 (4H, m), 8.02—8.23 (2H, m)
12fe	289 (M^+) 216 215 188	0.88 (6H, d, J=7), 1.84 (1H, sep, J=7), 2.75 (2H, t, J=7), 2.99—3.37 (6H, m), 3.60 (2H, t, J=7), 7.28—7.83 (4H, m), 8.01—8.12 (2H, m)
19hb	305 (M^+) 260 232 200	1.18 and 1.19 (6H, t, J=7), 2.77 (2H, t, J=7), 3.08 (1H, dd, J=14, 5), 3.24—3.65 (7H, m), 5.24 (1H, dd, J=7, 5), 7.58—7.83 (4H, m), 8.49—8.67 (2H, m)
20hb	464 (M^+) 232 (base)	1.15 (6H, t, J=7), 3.22—3.60 (8H, m), 5.40 (2H, t, J=7), 7.55—7.86 (8H, m), 8.45—8.62 (4H, m)
19ib	307 (M^++1) 261 233 201	1.18 and 1.19 (6H, t, J=7), 2.82 and 2.83 (2H, t, J=7), 3.15 (1H, dd, J=14, 5), 3.31—3.70 (7H, m), 5.30 (1H, dd, J=8, 5), 7.83—8.02 (3H, m), 8.67—8.77 (1H, m), 9.46 (1H, d, J=1)

TABLE III. High-Resolution MS of Photoproducts[a)]

Product	Formula	Calcd	Found	Product	Formula	Calcd	Found
8a	$C_{11}H_{17}NO_2S$	227.09811	227.09648	**11fb**	$C_{17}H_{23}NO_2S$	305.14509	305.14343
8b	$C_{13}H_{21}NO_2S$	255.12943	255.13092	**12fb**	$C_{15}H_{19}NOS$	261.11887	261.11781
9b	$C_{18}H_{24}N_2O_2S_2$	364.12812	364.12590	**11gb**	$C_{18}H_{25}NO_2S$	319.16080	319.16150
8c	$C_{15}H_{25}NO_2S$	283.16075	283.16280	**12gb**	$C_{16}H_{21}NOS$	275.13280	275.13450
9c	$C_{20}H_{28}N_2O_2S_2$	392.15944	392.16054	**11fc**	$C_{19}H_{27}NO_2S$	333.17641	333.18000
8d	$C_{17}H_{29}NO_2S$	311.19207	311.19390	**12fc**	$C_{16}H_{21}NOS$	275.13453	275.13447
9d	$C_{22}H_{32}N_2O_2S_2$	420.19076	420.19125	**11fd**	$C_{21}H_{31}NO_2S$	361.20773	361.21070
8e	$C_{17}H_{29}NO_2S$	311.19207	311.19326	**12fd**	$C_{17}H_{23}NOS$	289.15019	289.14918
9e	$C_{22}H_{32}N_2O_2S_2$	420.19076	420.19234	**11fe**	$C_{21}H_{31}NO_2S$	361.20773	361.20514
				12fe	$C_{17}H_{23}NOS$	289.15019	289.14844
				19hb	$C_{17}H_{23}NO_2S$	305.14509	305.14740
				19ib	$C_{16}H_{23}N_2O_2S$	307.14819	307.15018

a) Measurements for **9a** and **20hb** showed no parent peak.

General Procedure for the Photolysis——A solution of **1**, **10**, or **18** (1.0 mmol) with **7** (10 mmol) in 100 ml of solvent (CH_3CN for **1**, **10** and **18h**, CH_3OH for **18i**) was irradiated with a 500 W high-pressure mercury lamp through a Pyrex filter for 3—5 h (Table I) under an argon atmosphere. After removal of the solvent *in vacuo*, the residue was subjected to silica gel preparative layer chromatography (PLC) developed with hexane–acetone (solvent ratio), followed by distillation under reduced pressure. Spectral data are given in Tables II and III.

2-[1-Methoxy-2-(2methoxyethylthio)ethyl]pyridine (8a) and Bis[2-methoxy-2-(2-pyridyl)ethyl]disulfide (9a)——From 111 mg (1.0 mmol) of **1** and 600 mg (10 mmol) of **7a**. PLC (3:1). **8a**: bp 140 °C/0.3 mmHg, colorless oil, 44 mg (19%). **9a**: bp 230 °C/0.35 mmHg, colorless oil, 10 mg (3%).

2-[1-Ethoxy-2-(2-ethoxyethylthio)ethyl]pyridine (8b) and Bis[2-ethoxy-2-(2-pyridyl)ethyl]disulfide (9b)——From 111 mg (1.0 mmol) of **1** and 721 mg (10 mmol) of **7b**. PLC (3:1). **8b**: bp 160 °C/0.5 mmHg, colorless oil, 48 mg (19%). **9b**: bp 250 °C/0.8 mmHg, colorless oil, 14 mg (6%).

2-[1-Isopropoxy-2-(2-isopropoxyethylthio)ethyl]pyridine (8c) and Bis[2-isopropoxy-2-(2-pyridyl)ethyl]disulfide (9c)——From 111 mg (1.0 mmol) of **1** and 861 mg (10 mmol) of **7c**. PLC (5:1). **8c**: bp 175 °C/0.4 mmHg, colorless oil, 130 mg (46%). **9c**: bp 240 °C/0.6 mmHg, colorless oil, 12 mg (3%).

2-[1-*n*-Butoxy-2-(2-*n*-butoxyethylthio)ethyl]pyridine (8d) and Bis[2-*n*-butoxy-2-(2-pyridyl)ethyl]disulfide (9d)——From 111 mg (1.0 mmol) of **1** and 1.0 g (10 mmol) of **7d**. PLC (4:1). **8d**: bp 200 °C/0.7 mmHg, colorless oil, 184 mg (59%). **9d**: bp 250 °C/0.55 mmHg, colorless oil, 15 mg (7%).

2-[1-Isobutoxy-2-(2-isobutoxyethylthio)ethyl]pyridine (8e) and Bis[2-isobutoxy-2-(2-pyridyl)ethyl]disulfide (9e)——From 111 mg (1.0 mmol) of **1** and 1.0 g (10 mmol) of **7e**. PLC (4:1). **8e**: bp 167 °C/0.7 mmHg, colorless oil, 167 mg (54%). **9e**: bp 250 °C/0.6 mmHg, colorless oil, 22 mg (10%).

2-[1-Ethoxy-2-(2-ethoxyethylthio)ethyl]quinoline (11fb) and 2-[2-(2-Ethoxyethylthio)ethyl]quinoline (12fb)——From 161 mg (1.0 mmol) of **10f** and 721 mg (10 mmol) of **7b**. PLC (6:1). **11fb**: bp 210 °C/0.7 mmHg, colorless oil, 52 mg (17%). **12fb**: bp 180 °C/0.45 mmHg, colorless oil, 31 mg (12%). From 161 mg (1.0 mmol) of **10f** with 2.16 g (30 mmol) of **7b** by irradiation for 1 h, **11fb**: 69 mg (23%) and **12fb**: 35 mg (13%). The generated acetaldehyde was derived to the 2,4-dinitrophenylhydrazone: yellow needles, 24 mg (11%), mp 163—164 °C (lit.,[6)] mp 165—166 °C), identical in IR comparison and mixed melting point determination with an authentic sample.

2-[1-Ethoxy-2-(2-ethoxyethylthio)ethyl]-4-methylquinoline (11gb) and 2-[2-(2-Ethoxyethylthio)ethyl]-4-methylquinoline (12gb)——From 175 mg (1.0 mmol) of **10g** and 721 mg (10 mmol) of **7b**. PLC (7:1). **11gb**: bp 210 °C/0.3 mmHg, colorless oil, 45 mg (14%). **12gb**: bp 185 °C/0.3 mmHg, colorless oil, 29 mg (11%).

2-[1-Isopropoxy-2-(2-isopropoxyethylthio)ethyl]quinoline (11fc) and 2-[2-(2-Isopropoxyethylthio)ethyl]quinoline (12fc)——From 161 mg (1.0 mmol) of **10f** and 861 mg (10 mmol) of **7c**. PLC (7:1). **11fc**: bp 210 °C/0.3 mmHg, pale yellow oil, 50 mg (15%). **12fc**: bp 195 °C/0.7 mmHg, pale yellow oil, 68 mg (25%).

2-[1-*n*-Butoxy-2-(2-*n*-butoxyethylthio)ethyl]quinoline (11fd) and 2-[2-(2-*n*-Butoxyethylthio)ethyl]quinoline (12fd)——From 161 mg (1.0 mmol) of **10f** and 1.0 g (10 mmol) of **7d**. PLC (10:1). **11fd**: bp 220 °C/0.3 mmHg, pale yellow oil, 88 mg (24%). **12fd**: bp 200 °C/0.7 mmHg, pale yellow oil, 23 mg (8%).

2-[1-Isobutoxy-2-(2-isobutoxyethylthio)ethyl]quinoline (11fe) and 2-[2-(2-Isobutoxyethylthio)ethyl]quinoline (12fe)——From 161 mg (1.0 mmol) of **10f** and 1.0 g (10 mmol) of **7e**. PLC (10:1). **11fe**: bp 225 °C/0.6 mmHg, pale yellow oil, 52 mg (14%). **12fe**: bp 190 °C/0.4 mmHg, pale yellow oil, 76 mg (26%).

1-[1-Ethoxy-2-(2-ethoxyethylthio)ethyl]isoquinoline (19hb) and Bis[2-ethoxy-2-(1-isoquinolyl)ethyl]disulfide (20hb)——From 161 mg (1.0 mmol) of **18h** and 721 mg (10 mmol) of **7b**. PLC (aluminum oxide) (20:1). **19hb**: bp

200 °C/0.8 mmHg, colorless oil, 40 mg (13%). **20hb**: colorless oil (distillation was unsuccessful due to decomposition), 16 mg (7%).

1-[1-Ethoxy-2-(2-ethoxyethylthio)ethyl]phthalazine (19ib)——From 162 mg (1.0 mmol) of **18i** and 721 mg (10 mmol) of **7b**. PLC (15 : 1). bp 215 °C/0.9 mmHg, pale yellow oil, 69 mg (23%).

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Cycloadditions in Syntheses. XXXV.[1] 2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene and Its Derivatives: Synthesis and Reaction with Olefins

MASAYUKI SATO,[a] TAKESHI SUZUKI,[a] HIROKO MORISAWA,[a]
SHIGEO FUJITA,[b] NORIYOSHI INUKAI,[b]
and CHIKARA KANEKO*,[a]

Pharmaceutical Institute, Tohoku University,[a] Aobayama, Sendai 980, Japan and Central Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd.,[b] 1-8, Azusawa 1-chome, Itabashi-ku, Tokyo 174, Japan

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2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene was synthesized from 2-methoxynaphthalene *via* photoaddition to acrylonitrile followed by base treatment. The 4+2 cycloaddition of this compound to a variety of olefins by an application of the so-called benzocyclobutene method was examined and the regioselectivity and electron demand in these reactions were clarified. Construction of 12,13,14,15,16,17-hexahydro-11*H*-cyclopenta[*a*]phenanthrene derivatives having a basic A,B-ring-aromatized steroid skeleton from this naphthocyclobutene through either inter- or intramolecular 4+2 cycloaddition is also reported.

Keywords——2-cyanonaphtho[*a*]cyclobutene; naphtho[*a*]cyclobutene-2-carboxylic acid; benzocyclobutene method; 4+2 cycloaddition; photochemical synthesis; photo 2+2 addition; 11*H*-cyclopenta[*a*]phenanthrene; 1,2-dihydrocyclobuta[*a*]naphthalene; A,B-ring-aromatized steroid; cyclobutane annelation

The mode of photochemical reactions (addition and substitution) of aromatic compounds with olefins is affected by various factors, and is currently of considerable theoretical interest.[2] From a synthetic point of view, the 2+2 photocycloaddition reactions[3] are important, because these reactions provide a facile method for cyclobutane annelation to benzenes, naphthalenes, and their higher benzenoid homologues. A definite correlation exists between the electron-transfer energies of both reactants and the ease of the addition reactions: *i.e.*, the cycloaddition of benzene or naphthalene, substituted with a strong electron-donating or electron-withdrawing group, to alkenes proceeds efficiently only when the latter are oppositely substituted ethylenes. Among such reactions, the photoaddition of 2-methoxynaphthalene (an electron-rich arene) to acrylonitrile (an electron-deficient alkene) reported by McCullough *et al.*[4] proceeds regioselectively to give only the head-to-head adduct in very high yield. They also succeeded in synthesizing the corresponding 1,2-dihydrocyclobuta[*a*]naphthalene from the adduct by base-catalyzed elimination of methanol, though the yield in this step was poor and methanol elimination was accompanied with concomitant hydrolysis of the cyano group to a carbamoyl group. Since 1-cyanobenzocyclobutene and its derivatives[5] are the most useful compounds in the so-called benzocyclobutene method to construct complicated carbocyclic ring systems,[6] we are interested in developing an efficient synthetic method for so-far-unknown 2-cyano-1,2-dihydrocyclobuta[*a*]naphthalene and examining its further use in synthesis.

In this paper, we report a successful synthesis of this novel naphthocyclobutene and its derivatives, and we describe their inter- and intramolecular 4+2 cycloaddition reactions. This work provides a new methodology for the synthesis of A,B-ring-aromatized steroids.

Synthesis of 2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene and Its Derivatives

As reported by McCullough *et al.*,[4] when 2-methoxynaphthalene (**1**) was irradiated at ≥300 nm in the presence of an excess of acrylonitrile, the adduct (**2**: a mixture of *endo*- and *exo*-isomers in *ca.* 3:1 ratio) was obtained in *ca.* 90% yeild. Refluxing of the adduct (**2**) with potassium *tert*-butoxide in *tert*-butanol (the conditions used by McCullough *et al.*) afforded 2-carbamoyl-1,2-dihydrocyclobuta[*a*]naphthalene (**4**) in *ca.* 20% yield.

Though the photoaddition step proceeds within a few hours on a ten-gram scale, use of the carbamoyl derivative (**4**) as the starting material for synthetic work has two disadvantageous features: 1) the yield of **4** from the adduct (**2**) by McCullough's procedure was low and 2) this compound (**4**) is quite insoluble in most organic solvents, and so is not suitable for use in the benzocyclobutene method, in which benzocyclobutenes are necessarily heated in hydrocarbon solvents.

Hence, we first re-examined the base-catalyzed elimination of methanol from the adduct (**2**). As a result, it was found that use of dry benzene instead of *tert*-butanol as a solvent, and a reduction in the reaction time, resulted in a satisfactory result. Thus, refluxing of the adduct (**2**) with potassium *tert*-butoxide in dry benzene until disappearance of the starting material (20 min) resulted in the formation of the desired 2-cyano compound (**3**) in 48% yield. Though the carbamoyl derivative (**4**) was also obtained in 35% yield in the above reaction, separation of **3** from **4** was easily attained due to the insolubility of **4**. It is noteworthy that a longer reaction time after consumption of the starting material resulted in a decrease in the total amount of these naphthocyclobutenes (**3** and **4**) with an increase of the **4**/**3** ratio. If the whole products obtained in the above reaction were directly refluxed in a mixture of methanol and water (1:1, v/v) containing sodium hydroxide for 48 h (by this time, evolution of ammonia had ceased), the corresponding carboxylic acid (**5**) was obtained as a sole product in 84% overall yield from **2**. Treatment of **5** with diazomethane in ether afforded the corresponding methyl ester (**6**) in nearly quantitative yield.

Chart 1

Thus, we have accomplished a two-step synthesis of 1,2-dihydrocyclobuta[*a*]naphthalene having a cyano or methoxycarbonyl group at the 2-position from 2-methoxynaphthalene with satisfactory overall yields. These two naphthocyclobutenes (**3** and **6**) are soluble in most organic solvents, and hence are much more attractive materials than the carbamoyl derivative (**4**) for use in the benzocyclobutene method.

Intermolecular 4+2 Cycloaddition of 2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene (3) to Olefins

By analogy with the benzocyclobutene method, reaction of **3** (as a masked diene: A) with a variety of olefins in refluxing *o*-dichlorobenzene was examined. The olefins used were methyl

Chart 2

Table I. Products Obtained by Addition of 3 to Olefins

Olefin	Product (%)	
	4+2 Adduct	Dimer
Methyl methacrylate	7[a] (71)	None
n-Butyl vinyl ether	8[a] (91)	None
5-Bromo-1-pentene	9[a] (36)	10[b] (39)

a) A mixture of diastereomers. *b*) The other dimer was also obtained in 12% yield.

methacrylate (an electron-deficient alkene), *n*-butyl vinyl ether (an electron-rich alkene), and 5-bromo-1-pentene (an ordinary alkene). The reaction conditions employed were as follows: a 0.18 M solution of **3** in *o*-dichlorobenzene containing 20 mol equivalents of olefins was heated at reflux until all of **3** was consumed. The products obtained from these reactions are listed in Table I.

The yield of 4+2 adducts (**7**, **8**, and **9**) depended upon the kind of olefins used. Thus, while **3** reacts efficiently with either electron-deficient or electron-rich alkenes to give the 4+2 adducts (**7** and **8**) in high yields, the yield of the adduct (**9**) was poor and in this case, two dimers (**10** and the other) were obtained in yields of 39% and 12%, respectively. Thus, the reactivity of **3** is less for an ordinary alkene (5-bromo-1-pentene) than for the electron-deficient or electron-rich alkenes. Rate enhancement by the introduction of substituents (electron-donating as well as electron-withdrawing) on the alkene (a dipolarophile or dienophile) was observed in the 1,3-dipolar cycloaddition of benzonitrile oxide[7] and phenyl

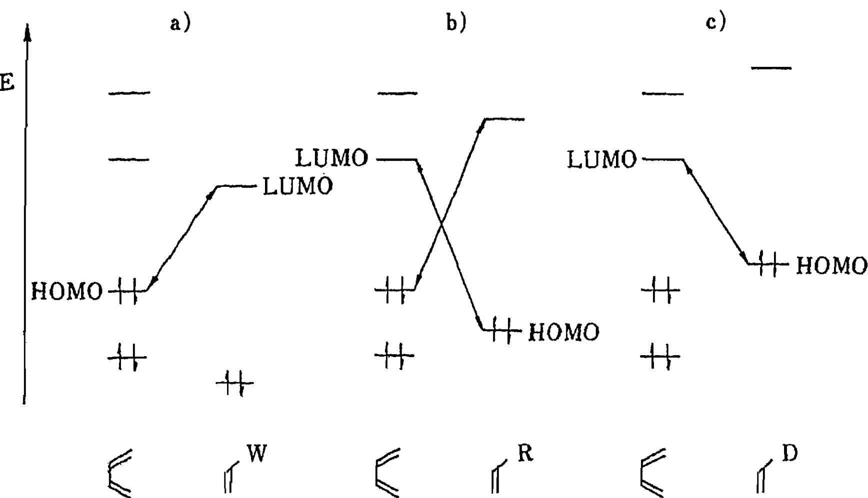

Fig. 1. HOMO–LUMO Arrangements for the 4+2 Addition of Diene (A) to Olefins

W is an electron-withdrawing substituent and D is an electron-donating substituents.

azide,[8] and also in Diels–Alder reaction of tetraphenylcyclopentadienone.[9]

Three possible arrangements of highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs) of dienes and dienophiles in such Diels–Alder reactions are shown in Fig. 1.[10]

Taking case b [reaction of the diene (**3**) with 5-bromo-1-pentene] as the standard, introduction of an electron-withdrawing group or electron-donating group into the alkene results in a smaller energy difference between $HOMO_{diene}$–$LUMO_{dienophile}$ (case a in Fig. 1: normal Diels–Alder reaction) or between $HOMO_{dienophile}$–$LUMO_{diene}$ (case c: Diels–Alder reaction with inverse electron demand), and hence accelerates the formation of the adducts (**7** and **8**).

Formation of the dimer (**10**) by reaction of **3** with the pentene can be explained, if one considers that the cyanomethylene group in the intermediate (A) acts as a better dienophile than the simple alkene (compare cases a and b). The nuclear magnetic resonance (NMR) spectrum of **10** clearly indicates the head-to-head structure [the methine and methylene protons in the 8-membered ring appeared at δ 4.88 (2H) and 3.85 (4H) as broad singlets, respectively] and is inconsistent with the head-to-tail structure (**10'**).[11] The formation of **10** may proceed *via* the 4+2 adduct (B) as the primary product. The arguments given above are consistent with the observed regio-selectivity, *i.e.*, formation of the head-to-head adducts (**7**, **8**, and **9**) in all cases.

B CN CN   C Z CN

Though the above arguments are based on the assumption that these 4+2 addition reactions proceed in a concerted manner, the regio-selectivity in these addition reactions is also explainable in terms of a stepwise process involving the biradical species (C) as an intermediate.

While the mechanistic details in these addition reactions have not yet been clarified

completely, it has been verified experimentally that the quinodimethane species (A) derived from **3** affords the 4+2 adducts in high yields with both electron-rich and electron-deficient alkenes and the adducts always have the head-to-head structure. Thus, **3** should be a useful synthon for the construction of 1,2-substituted 1,2,3,4-tetrahydrophenanthrene derivatives.

Treatment of **9** with base afforded 14-cyano-12,13,14,15,16,17-hexahydro-11*H*-cyclopenta[*a*]phenanthrene (**11**) in quantitative yield. Since **9** is a *ca.* 1 : 1 mixture of *cis–trans* isomers, the C/D ring junction of **11** is considered to be *cis* due to favorable kinetic control in the cyclization step. This assumption was verified by X-ray crystallographic analysis of **11** (*vide infra*).

Synthesis of A,B-Ring-Aromatized Steroidal Compounds from 3 and 6 *via* Intramolecular 4+2 Cycloaddition Reactions

In order to elaborate a new synthetic route to A,B-ring-aromatized steroids (*e.g.* D) from the newly synthesized naphthocyclobutenes (**3** and **6**), we chose compound F as a suitable intermediate. This is because F (irrespective of the stereochemistry) is expected to give E on oxidative decarboxylation[12] which in turn affords D on catalytic hydrogenation.

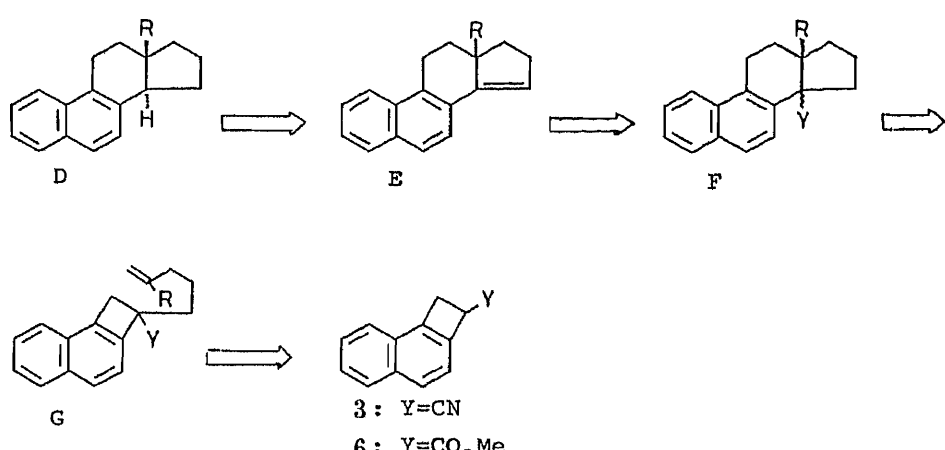

Chart 3. Retrosynthesis of A,B-Ring-Aromatized Steroids
Y represents CO_2H or its equivalent.

Though we have already succeeded in the synthesis of **11** (nor-methyl derivative of F) by base-catalyzed cyclization of **9** (*vide supra*), its overall yield from **3** was poor. Therefore, a more efficient route to **11** and its 13-methyl derivative (**12**) from **3** through intramolecular cycloaddition of G was examined. Due to favorable entropy assistance in such intramolecular Diels–Alder reactions, the addition step (G→F) is expected to be efficient even if the ene function in G is a simple alkene.

Treatment of **3** with 5-bromo-1-pentene or its 2-methyl derivative under basic conditions afforded the corresponding 2-alkylated compounds (**13** and **14**). Refluxing of **13** in *o*-dichlorobenzene resulted in the exclusive formation of **11**. The structure of **11** was identified by mixed melting point determination with the sample obtained by base-catalyzed cyclization of **9**, and **11** was confirmed to be the C/D-*cis*-isomer by X-ray analysis (Fig. 2). The same stereoselectivity was also observed for cyclization of **14**. The corresponding tetracyclic products were deduced to be stereoisomeric mixtures ($\mathbf{12}_{cis}$ and $\mathbf{12}_{trans}$: *ca.* 10 : 1 ratio) from the ^{1}H-NMR spectra, and the major isomer was assigned as the C/D-*cis*-isomer ($\mathbf{12}_{cis}$), because the 13-methyl group in the major adduct showed a proton signal at δ 1.31 which is at lower field than that (δ 0.79) of the minor adduct ($\mathbf{12}_{trans}$).[13] Predominance of the *cis* isomers ($\mathbf{11}_{cis}$ and $\mathbf{12}_{cis}$) over the *trans* isomers indicates a preference for the *endo* transition state

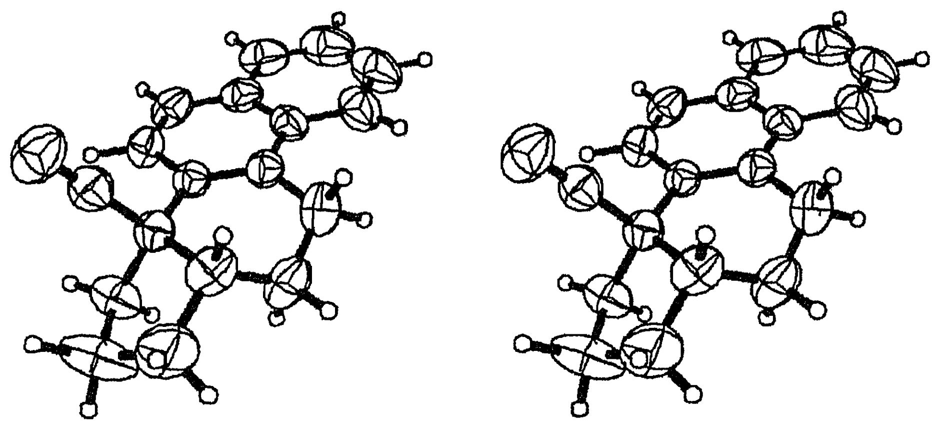

Fig. 2. Stereoview of $\mathbf{11}_{cis}$

Thermal ellipsoids are drawn at the 50% probability level.

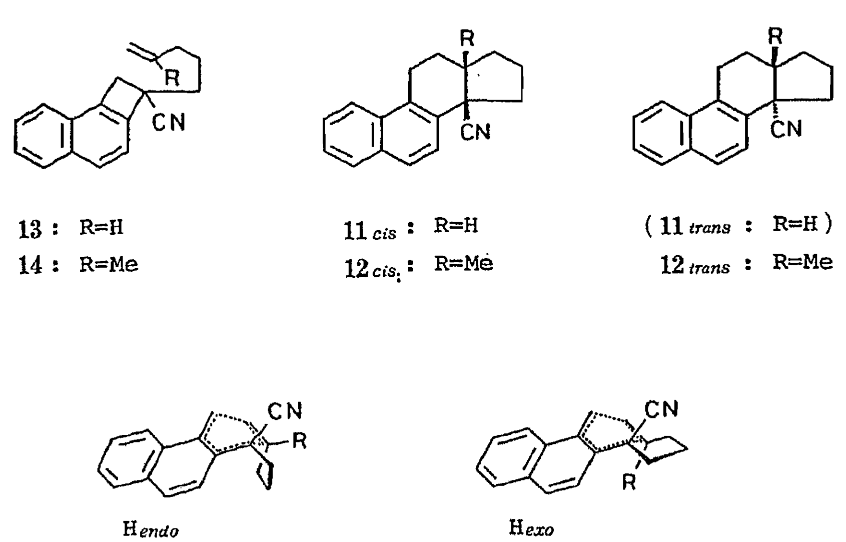

Chart 4

(H_{endo}) over the *exo* transition state (H_{exo}).

Though the hydrolysis of these tetracyclic compounds (**11** and **12**) was examined under a variety of conditions (either basic or acidic), none of the corresponding acid [F (C/D-*cis*): Y = CO_2H] was obtained and, in all cases, the starting material was recovered. Thus, in order to synthesize these acids (F: Y = CO_2H), use of the ester (**6**) instead of **3** as the starting material in the above transformation was examined. When **6** was treated with 5-bromo-1-pentene under the same conditions as in the case of **3** until disappearance of the starting material, several compounds (**15**, **16**, and **17**) were obtained, together with a small amount (7%) of the simple hydrolysis product (**5**). It was found, however, that further exposure of this product mixture to the same conditions resulted in exclusive formation of **17**, with the consumption of both **15** and **16**. Methylation of the acid (**17**) afforded the desired **15** in 75% overall yield from **6**. By heating of **15** in *o*-dichlorobenzene, the tetracyclic compound (**18**) was obtained exclusively; its stereochemistry was assigned as *cis* by analogy to the conversion of **13** to $\mathbf{11}_{cis}$ *via* the *endo* cyclization. Hydrolysis of **18** then afforded the carboxylic acid (**19**) in quantitative yield. It should be noted that when **17** was cyclized under the same conditions, no **19** was obtained and the sole product was 4-(4-pentenyl)-3-oxo-3,4-dihydro-1*H*-naphtho[1,2-*c*]pyran (**20**), which clearly arose from 6π-electrocyclic ring closure of I.[14]

15 16 17

18 : R=Me
19 : R=H
I
20

Chart 5

Conclusion

By utilizing the 2+2 photoadduct obtained from 2-methoxynaphthalene and acrylonitrile, 2-cyano-1,2-dihydrocyclobuta[*a*]naphthalene (**3**) and its derivatives (*e.g.* **6**) were synthesized in satisfactory overall yields. Heating of **3** in *o*-dichlorobenzene in the presence of olefins afforded 1,2-substituted 1,2,3,4-tetrahydrophenanthrenes. Finally, a facile method for the construction of C/D-*cis*-steroidal compounds (*e.g.* **19**) has been developed through intramolecular 4+2 cycloaddition of 2-(4-pentenyl) derivatives of **6**. Further elaboration of the C/D-*cis* isomer to the C/D-*trans* isomer is in progress, and should allow even more general application of this reaction to construct polycyclic ring systems containing at least one naphthalene ring.

Experimental

All melting points were determined on a micro-hot stage (Yanagimoto) and are uncorrected. Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer, ultraviolet (UV) spectra with a Hitachi 320 spectrometer, and ^{1}H-NMR spectra on a JEOL JNM-PMX60SI or JEOL JNM-FX-100 spectrometer (with tetramethylsilane as an internal standard). Mass spectra (MS) were taken either with a Hitachi M-52 spectrometer or with a JEOL JMS-01SG-2 spectrometer. Silica gel used for column chromatography was Wakogel C-200. Preparative thin-layer chromatography (PTLC) was performed on Merck Kiesel-gel 60 F254. The photolysis was carried out in a Pyrex immersion apparatus equipped with a RIKO 1 kW high-pressure mercury lamp.

Photoaddition of 2-Methoxynaphthalene (1) to Acrylonitrile——A solution of 2-methoxynaphthalene (10 g, 63 mmol) and acrylonitrile (30 g, 570 mmol) in methanol (2000 ml) was irradiated for 1.5 h. The residue obtained after evaporation of the solvent *in vacuo* was chromatographed on silica gel (200 g). Elution with hexane–ethyl acetate (20 : 1) gave 4.60 g (46%) of the unreacted naphthalene (**1**). Elution with hexane–ethyl acetate (5 : 1) gave 6.50 g [49% (91% based on the consumed **1**)] of **2** as a semi-solid.

McCullough and Chamberlin[4a)] carried out this reaction in ethanol and obtained the same adduct (*endo*/*exo* ratio: *ca.* 3 : 1) in 71% yield (based on **1**). Furthermore, they succeeded in the separation of each isomer by column chromatography using a large amount of silica gel and tentatively assigned the stereo-structures of each isomer by ^{1}H-NMR spectroscopy. Based on their ^{1}H-NMR data, the ratio of *endo*- and *exo*-isomers of our adduct (**2**) was determined as *ca.* 3 : 1 from the relative peak areas of vinylic proton signals [*endo*: δ 6.92 (d, J=10 Hz) and 5.83 (dd, J=10.0, 1.5 Hz) and *exo*: δ 6.82 (d, J=10 Hz) and 5.65 (dd, J=10.0, 1.0 Hz)].

In our case, the adduct (a mixture of *endo*- and *exo*-isomers) was used for further studies without separation of the isomrs.

2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene (3)——A solution of the adduct (530 mg, 2.5 mmol) in benzene (5 ml) containing *tert*-BuOK (560 mg, 5.0 mmol) was heated at reflux for 20 min. Then 10% hydrochloric acid was added to the reaction mixture under ice-cooling, and the precipitate (140 mg of the 2-carbamoyl derivative) was filtered off. The filtrate was extracted with ether and the organic layer was separated and dried over $MgSO_4$. The

residue obtained after evaporation of the solvent was chromatographed on silica gel (30 g). Elution with hexane–ethyl acetate (20 : 1) gave 215 mg (48%) of **3**. Elution with hexane–ethyl acetate (1 : 1) afforded a further amount (33 mg) of the carbamoyl derivative. The total amount of the carbamoyl derivative (**4**) was 173 mg (35%).

3: Colorless needles, mp 105—106 °C (hexane–ether). IR ($CHCl_3$): 2240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.69 (1H, dd, J=13.5, 3.0 Hz), 3.89 (1H, dd, J=13.5, 4.0 Hz), 4.33 (1H, dd, J=4.0, 3.0 Hz), 7.15—8.10 (6H, m). *Anal.* Calcd for $C_{13}H_9N$: C, 87.12; H, 5.06; N, 7.82. Found: C, 87.09; H, 4.98; N, 7.86. MS *m/z*: 179 (M^+).

4: Colorless needles, mp 215—219 °C (MeOH) (lit.[4a] mp 202—204 °C). ^{1}H-NMR ($CDCl_3$–DMSO-d_6, 2 : 1) δ 3.59 (2H, d, J=3.5 Hz), 4.32 (1H, t, J=3.5 Hz), 6.67 (2H, br s), 7.0—8.0 (6H, m).

1,2-Dihydrocyclobuta[*a*]naphthalene-2-carboxylic Acid (5)——A solution of the adduct (584 mg, 2.8 mmol) in benzene (5 ml) containing *tert*-BuOK (630 mg, 5.6 mmol) was refluxed for 25 min. The residue obtained after evaporation of the solvent was dissolved in a mixture of methanol (10 ml) and water (10 ml) containing sodium hydroxide (3 g). The whole was refluxed for 48 h (by this time, evolution of ammonia had ceased). After evaporation of the solvent *in vacuo*, water (10 ml) was added and the whole was washed with ether. The aqueous layer was separated and acidified by the addition of 10% hydrochloric acid and extracted with ether. The ethereal layer was washed with water and dried over $MgSO_4$. The crystalline residue obtained after evaporation of the solvent was recrystallized from hexane–ether to give 485 mg (84%) of **5**. Colorless needles, mp 181—182 °C (hexane–ether). IR ($CHCl_3$): 3400—2400, 1705 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.66 (2H, d, J=3.5 Hz), 4.36 (1H, t, J=3.5 Hz), 7.1—8.0 (6H, m), 10.3 (1H, br s). *Anal.* Calcd for $C_{13}H_{10}O_2$: C, 78.77; H, 5.08. Found: C, 78.60; H, 4.93. MS *m/z*: 198 (M^+).

Methyl 1,2-Dihydrocyclobuta[*a*]naphthalene-2-carboxylate (6)——An ethereal solution of diazomethane was added to a solution of **5** (100 mg) in ether until the yellow color of the reaction mixture was maintained. After 30 min, the ether was evaporated off and the residue was chromatographed on silica gel. Elution with hexane–ethyl acetate (10 : 1) gave 101 mg (94%) of **6**. Colorless prisms, mp 48—49 °C (pentane). IR ($CHCl_3$): 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.62 (2H, d, J=3.5 Hz), 3.67 (3H, s), 4.36 (1H, t, J=3.5 Hz), 7.15—7.95 (6H, m). *Anal.* Calcd for $C_{14}H_{12}O_2$: C, 79.23; H, 5.70. Found: C, 79.04; H, 5.56. MS *m/z*: 212 (M^+).

General Procedure for the Intermolecular 4+2 Cycloaddition of 2-Cyano-1,2-dihydrocyclobuta[*a*]naphthalene (3) to Olefins——Use of Methyl Methacrylate as the Olefin as a Typical Example: A solution of **3** (125 mg, 0.70 mmol) and methyl methacrylate (1.41 g, 20 mol eq to **3**) in *o*-dichlorobenzene (4 ml) was refluxed for 4 h. The residue obtained after evaporation of the solvent was chromatographed on silica gel. Elution with hexane–ethyl acetate (10 : 1) afforded the 4+2 adduct as a mixture of diastereoisomers (**7a** and **7b**). Separation of the adducts by PTLC [hexane–ether (20 : 1)] gave 91 mg (47%) of the major adduct (**7a**) and 46 mg (24%) of the minor adduct (**7b**). The head-to-head structure of both adducts was confirmed by their ^{1}H-NMR spectra.

7a: Colorless oil. IR ($CHCl_3$): 2250, 1732 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.55 (3H, s), 1.9—2.7 (2H, m), 3.21 (2H, t, J=6.0 Hz), 3.61 (3H, s), 4.60 (1H, s), 7.25—8.05 (6H, m). High-resolution MS *m/z*: M^+ Calcd for $C_{18}H_{17}NO_2$: 279.1258. Found: 279.1259.

7b: Colorless prisms, mp 135—136 °C (hexane–ether). IR ($CHCl_3$): 2250, 1730 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.27 (3H, s), 1.9—2.8 (2H, m), 3.23 (2H, t, J=6.0 Hz), 3.76 (3H, s), 4.10 (1H, s), 7.2—8.15 (6H, m). *Anal.* Calcd for $C_{18}H_{17}NO_2$: C, 77.40; H, 6.13; N, 5.01. Found: C, 77.24; H, 6.05; N, 5.00. MS *m/z*: 279 (M^+).

Use of *n*-butyl vinyl ether afforded the 4+2 adducts (**8a** and **8b**).

8a (69%): Colorless oil. IR ($CHCl_3$): 2250, 1100 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.70—1.15 (3H, m), 1.15—1.95 (4H, m), 1.95—2.40 (2H, m), 2.65—3.45 (2H, m), 3.62 (2H, t, J=6.5 Hz), 3.77—4.05 (1H, m), 4.27 (1H, d, J=4.5 Hz), 7.18—8.10 (6H, m). High-resolution MS *m/z*: M^+ Calcd for $C_{19}H_{21}NO$: 279.1623. Found: 279.1603.

8b (23%): Colorless prisms. mp 85—86 °C (hexane–ether). IR ($CHCl_3$): 2250, 1097 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, t, J=6.0 Hz), 1.05—1.75 (4H, m), 1.75—2.20 (1H, m), 2.25—2.60 (1H, m), 2.90—3.83 (4H, m), 3.83—4.20 (1H, m), 4.07 (1H, s), 7.40—8.10 (6H, m). *Anal.* Calcd for $C_{19}H_{21}NO$: C, 81.64; H, 7.58; N, 5.01. Found: C, 81.54; H, 7.54; N, 4.92. MS *m/z*: 279 (M^+).

Use of 5-bromo-1-pentene afforded, after separation by silica gel column chromatography [hexane–ethyl acetate (10 : 1)], the 4+2 adduct (**9**) in 36% yield as an inseparable 1 : 1 mixture of diasteroisomers. Further elution with hexane–ethyl acetate (4 : 1) afforded 51 mg (39%) of dimer (**10**: 39%) and then 16 mg (12%) of the other dimer (12%), whose structure was not determined.

9: Colorless oil. IR ($CHCl_3$): 2240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30—2.50 (7H, m), 2.80—3.60 (4H, m), 3.84 (0.5H, d, J=8.0 Hz), 4.05 (0.5H, s), 7.20—8.10 (6H, m). High-resolution MS *m/z*: M^+ Calcd for $C_{18}H_{18}BrN$: 327.0623 (for ^{79}Br), 329.0603 (for ^{81}Br). Found: 327.0607, 329.0555.

10: Colorless prisms, mp 275—278 °C (hexane–ether). IR ($CHCl_3$): 2250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.85 (4H, br s), 4.88 (2H, br s), 7.00—7.70 (10H, m), 7.80—8.15 (2H, m). *Anal.* Calcd for $C_{26}H_{18}N_2$: C, 87.12; H, 5.06; N, 7.82. Found: C, 86.89; H, 4.92; N, 7.78. MS *m/z*: 358 (M^+).

Other Dimer: Colorless prisms, mp 264—267 °C (MeOH–CH_2Cl_2). MS *m/z*: 358.

Synthesis of 2-Cyano-2-(ω-alkenyl)-1,2-dihydrocyclobuta[*a*]naphthalenes (13 and 14)——Synthesis of 2-Cyano-2-(4-pentenyl)-1,2-dihydrocyclobuta[*a*]naphthalene (**13**) as a Typical Example: Sodium hydride (41 mg, 1.7 mmol) was added to a solution of **3** (75 mg, 0.42 mmol) in dimethylformamide (DMF), and the mixture was stirred for 20 min. Then 5-bromo-1-pentene (98 mg, 0.63 mmol) was added and the whole was stirred for 2 h at room temperature. After

addition of ice-water to the reaction mixture, the product was taken up in ether. The organic layer was washed with water and dried over $MgSO_4$. The residue obtained after evaporation of the solvent was chromatographed on silica gel (10 g). Elution with hexane–ethyl acetate (20 : 1) gave 82 mg (80%) of **13**. Colorless oil. IR ($CHCl_3$): 2240 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.45—2.45 (6H, m), 3.44 (1H, d, J=14.0 Hz), 3.93 (1H, d, J=14.0 Hz), 4.80—5.30 (2H, m), 5.45—6.20 (1H, m), 7.20—8.05 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{18}H_{17}N$: 247.1361. Found: 247.1351.

2-Cyano-2-(4-methyl-4-pentenyl)-1,2-dihydrocyclobuta[*a*]naphthalene (**14**) was prepared from **3** and 5-iodo-2-methyl-1-pentene in the same manner.

14: Yield, 73%. Colorless oil. IR ($CHCl_3$): 2240 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.20—2.50 (6H, m), 1.73 (3H, br s), 3.43 (1H, d, J=14.0 Hz), 3.94 (1H, d, J=14.0 Hz), 4.71 (1H, br s), 7.10—8.10 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{19}H_{19}N$: 261.1517. Found: 261.1513.

(13*S,14*R**)-2-Cyano-12,13,14,15,16,17-hexahydro-11*H*-cyclopenta[*a*]phenanthrene (11_{cis})**——(a) From **13**: A solution of **13** (21 mg, 0.085 mmol) in *o*-dichlorobenzene (2 ml) was refluxed for 7 h. The residue obtained after evaporation of the solvent was separated by PTLC (hexane–AcOEt, 50 : 1) to give 14 mg (67%) of 11_{cis}. Colorless prisms, mp 111—112 °C (MeOH–H_2O). IR ($CHCl_3$): 2245 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.30—3.00 (9H, m), 3.17 (2H, br t, J=6.0 Hz), 7.35—8.20 (6H, m). *Anal.* Calcd for $C_{18}H_{17}N$: C, 87.41; H, 6.93; N, 5.66. Found: C, 87.15; H, 6.86; N, 5.58. MS m/z: 247 (M^+).

(b) From **9**: Sodium hydride (8.4 mg, 0.35 mmol) was added to a solution of **9** (30 mg, 0.09 mmol) in DMF (1 ml) and the whole was stirred at room temperature for 4 h. After addition of water (15 ml), the product was taken up in ether and dried over $MgSO_4$. The residue obtained after evaporation of the solvent was separated by PTLC [hexane–ethyl acetate (20 : 1)] to give 18 mg (80%) of 11_{cis}. This compound was identical with the sample obtained in (a) by mixed melting point determination.

Crystallographic Measurements of 11_{cis}——Colorless prismatic crystals of 11_{cis} were grown in water–methanol solution. A crystal, 0.3 × 0.3 × 0.07 mm^3 in size, was used for data collection on a Rigaku AFC-5R diffractometer with graphite-monochromated CuK_α radiation (λ=1.54184 Å). The crystal data are as follows: $C_{18}H_{17}N$, M.W. 247.34, orthorhombic, space group *Pbcn*, a=13.268 (1), b=9.882 (1), c=20.724 (2) Å and Z=8. Intensities were measured in the $\omega/2\theta$ scan mode with a scan speed of 4° (2θ)/min. Of 2171 independent reflections with $2\theta<126°$, 291 weak reflections below the background were considered to be zero reflections. Corrections were made for Lorentz and polarization factors but not for absorption. The standard deviations were estimated by using the equation $\sigma(|F_o|)=\sigma_p^2(|F_o|)+q^2F_o^2$, where q, 0.0126, was derived from the variation of 5 reflections monitored after every 50 reflections and σ_p was due to the counting statistics.[16]

The structure was solved by the direct method[17] and the atomic parameters were refined by the block-diagonal least-squares method. All hydrogen atoms were found on a difference map and were included in the refinement. The quantity minimized was $\Sigma\omega(|F_o|-|F_c|)^2$ with $\omega=1/\sigma(|F_o|)$. In the refinement, the zero reflections with $|F_c|>F_{lim}$ were included by assuming $F_o=F_{lim}$ and $\omega=\omega(F_{lim})$, F_{lim}, 0.056, being the observed threshold value. The final R value was 0.060 for 1487 reflections with $|F_o|>3$ ($|F_o|$). The atomic parameters, F_o-F_c table, distances and bond angles have been deposited. The atomic scattering factors were taken from reference 18.

Intramolecular [4+2] Cycloaddition Reaction of 2-Cyano-2-(4-methyl-4-pentenyl)-1,2-dihydrocyclobuta[*a*]-naphthalene (14)——A solution of **14** (103 mg, 0.39 mmol) in *o*-dichlorobenzene (10 ml) was refluxed for 8 h. The residue obtained after evaporation of the solvent *in vacuo* was chromatographed on silica gel (35 g). Elution with hexane–ethyl acetate (40 : 1) gave the adduct (**12**) as a mixture of diastereoisomers. This mixture was separated by PTLC [hexane–ethyl acetate (50 : 1)] to afford 65 mg (63%) of 12_{cis} and 6 mg (6%) of 12_{trans}.

12_{cis}: Colorless prisms, mp 176—178 °C (hexane–CH_2Cl_2). IR ($CHCl_3$): 2245 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.31 (3H, s), 1.55—3.00 (8H, m), 3.14 (2H, t, J=6.5 Hz), 7.15—8.20 (6H, m). *Anal.* Calcd for $C_{19}H_{19}N$: C, 87.31; H, 7.33; N, 5.36. Found: C, 87.10; H, 7.05; N, 5.33. MS m/z: 261 (M^+).

12_{trans} (contaminated with a small amount of 12_{cis}): Semisolid. ^1H-NMR ($CDCl_3$) δ: 0.79 (3H, s), 3.38 (2H, dt, J=8.0, 2.0 Hz).

Attempted Hydrolysis of 12_{cis}——(a): A solution of 12_{cis} (20 mg) in ethylene glycol (1 ml) and 10% aq. KOH (3 ml) was refluxed for 10 h. After usual work-up, 12_{cis} was recovered quantitatively.

(b): A solution of 12_{cis} (20 mg) in a mixture of acetic acid (1 ml) and concentrated hydrochloric acid (1 ml) was refluxed for 10 h. After usual work-up, 12_{cis} was recovered quantitatively.

Reaction of 6 with 5-Bromo-1-pentene——Sodium hydride (101 mg, 4.2 mmol) was added to a solution of **6** (294 mg, 1.39 mmol) and 5-bromo-1-pentene (330 mg, 2.1 mmol) in DMF (3 ml) and the whole was stirred at room temperature for 6 h. After addition of ice-water, the product was taken up in ether. The organic layer was dried over $MgSO_4$. The residue obtained after evaporation of the ether was chromatographed on silica gel (25 g). Elution with hexane–ethyl acetate (20 : 1) gave 16 mg (5%) of **16** and 198 mg (51%) of **15**. The aqueous layer was acidified by the addition of 10% HCl (ice-cooling) and the product was taken up in ether. The ether layer was dried over $MgSO_4$. The residue obtained after evaporation of the solvent was chromatographed on silica gel (15 g). Elution with hexane–ethyl acetate (5 : 1) gave 61 mg (16%) of **17**. Elution with hexane–ethyl acetate (3 : 1) gave 19 mg (7%) of **5**.

16: Colorless oil. IR ($CHCl_3$): 1718 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.20—2.60 (10H, m), 3.30 (1H, d, J=14.0 Hz),

3.87 (1H, d, J=14.0 Hz), 4.13 (2H, t, J=6.5 Hz), 4.75—5.25 (4H, m), 5.45—6.30 (2H, m), 7.20—8.20 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{23}H_{26}O_2$: 334.1933. Found: 334.1920.

15: Colorless oil. IR ($CHCl_3$): 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20—1.90 (2H, m), 1.90—2.40 (4H, m), 3.33 (1H, d, J=14.0 Hz), 3.70 (3H, s), 3.90 (1H, d, J=14.0 Hz), 4.80—5.25 (2H, m), 5.45—6.25 (1H, m), 7.20—8.10 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{19}H_{20}O_2$: 280.1463. Found: 280.1487.

17: Colorless prisms, mp 95—96 °C (hexane). IR ($CHCl_3$): 3200—2400, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.10—1.85 (2H, m), 1.85—2.40 (4H, m), 3.30 (1H, d, J=14.0 Hz), 3.89 (1H, d, J=14.0 Hz), 4.75—5.25 (2H, m), 5.40—6.20 (1H, m), 7.15—8.05 (6H, m), 11.50 (1H, br s). *Anal.* Calcd for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81. Found: C, 80.95; H, 6.80. MS m/z: 266 (M^+).

When the above pentenylation reaction was continued for 15 h, **17** was obtained in 81% yield. Neither **15** nor **16** was isolated in this case.

2-Methoxycarbonyl-2-(4-pentenyl)-1,2-dihydrocyclobuta[*a*]naphthalene (15)——Usual methylation of **17** with diazomethane afforded **15** in quantitative yield. The product was identical with the sample obtained above.

14-Methoxycarbonyl-12,13,14,15,16,17-hexahydro-11*H*-cyclobuta[*a*]phenanthrene (18)——A solution of **15** (138 mg, 0.49 mmol) in *o*-dichlorobenzene (15 ml) was refluxed for 16 h. The residue obtained after evaporation of the solvent was chromatographed on silica gel (35 g). Elution with hexane–ethyl acetate (20 : 1) affored 127 mg (92%) of **18**.[19] Colorless oil. IR ($CHCl_3$): 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30—2.40 (6H, m), 2.45—2.95 (2H, m), 3.13 (2H, t, J=6.0 Hz), 3.58 (3H, s), 7.15—8.20 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{19}H_{20}O_2$: 280.1464. Found: 280.1478.

14-Carboxy-12,13,14,15,16-hexahydro-11*H*-cyclopenta[*a*]phenanthrene (19)——A solution of **15** in a mixture of 15% aq. NaOH (10 ml) and methanol (5 ml) was refluxed for 10 h. After evaporation of the solvent and addition of water (15 ml), the whole was washed with ether, twice. After acidification of the aqueous layer with 10% HCl, the product was taken up in ether. The ethereal layer was dried over $MgSO_4$. Evaporation of ether gave 71 mg (96%) of **19**. Colorless needles, mp 147—148 °C (hexane–ether). IR ($CHCl_3$): 3200—2400, 1692 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.15—2.45 (6H, m), 2.65—2.95 (2H, m), 3.10 (2H, t, J=6.0 Hz), 7.10—8.15 (6H, m), 11.72 (1H, br s). *Anal.* Calcd for $C_{18}H_{18}O_2$: C, 81.17; H, 6.81. Found: C, 81.25; H, 6.93. MS m/z: 266 (M^+).

4-(4-Pentenyl)-3-oxo-3,4-dihydro-1*H*-naphtho[1,2-*c*]pyrane (20)——A solution of **17** (32 mg, 0.12 mmol) in *o*-dichlorobenzene (2 ml) was refluxed for 2.5 h. The residue obtained after evaporation of the solvent *in vacuo* was chromatographed on silica gel (10 g). Elution with hexane–ethyl acetate (10 : 1) gave 30 mg (94%) of **20**. Colorless oil. IR ($CHCl_3$): 1735 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.10—2.40 (6H, m), 3.81 (1H, t, J=6.5 Hz), 4.75—5.25 (2H, m), 5.40—6.20 (1H, m), 5.86 (2H, br s), 7.18—8.05 (6H, m). High-resolution MS m/z: M^+ Calcd for $C_{18}H_{18}O_2$: 266.1307. Found: 266.1307.

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Syntheses of Optically Active 9-Hydroxymethyl- and 9-Carbamoyloxymethyl-9-deacetyl-4-demethoxydaunomycinone[1]

MICHIYO SUZUKI, TERUYO MATSUMOTO, MASAKO OHSAKI, YOSHIKAZU KIMURA, and SHIRO TERASHIMA*

Sagami Chemical Research Center, 4-4-1, Nishi-Ohnuma, Sagamihara, Kanagawa 229, Japan

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Reduction of (*R*)-methyl 2,5,12-trihydroxy-6,11-dioxo-1,2,3,4-tetrahydronaphthacene-2-carboxylate ((*R*)-**5**) with lithium tri-*tert*-butoxyaluminum hydride in dimethyl sulfoxide was found to proceed chemoselectively, giving the corresponding alcohol ((*R*)-**11**) in 50—55% yield. The produced (*R*)-alcohol ((*R*)-**11**) could be readily isolated as its acetonide ((*R*)-**12**) or *tert*-butyldimethylsilyl ether ((*R*)-**13**). Stereoselective $C_{7\alpha}$-hydroxylation (the anthracycline numbering) of (*R*)-**13** and urethane formation produced the optically active title compounds, which are the aglycones of unnatural anthracyclines showing excellent anticancer activity.

Keywords——anthracyclinone; 9-deacetyl-anthracyclinone; 9-hydroxymethyl-9-deacetyl-4-demethoxydaunomycinone; 9-carbamoyloxymethyl-9-deacetyl-4-demethoxydaunomycinone; chemoselective reduction; dimethyl sulfoxide; lithium tri-*tert*-butoxyaluminum hydride; $C_{7\alpha}$-hydroxylation; isocyanate; urethane formation

The anthracycline antibiotics, represented by adriamycin (**1a**) and daunorubicin (**1b**), are clinically useful antitumor agents which have recently been the subjects of much synthetic endeavor.[2,3] Attempts have been made to prepare various structural types of congeners by chemical synthesis or by modification of natural anthracyclines with the aim of finding unnatural anthracyclines which show superior therapeutic properties to natural **1a**, **b**.[2,4,5]

Among synthetically elaborated analogues of **1a**, **b**, 4-demethoxyadriamycin (**1c**) and 4-demethoxydaunorubicin (**1d**) are well known to exhibit better therapeutic indices than **1a**, **b**[6] and are currently under clinical trials.[7] We are interested in 9-hydroxymethyl-9-deacetyl-4-demethoxydaunorubicin (**1e**) and 9-carbamoyloxymethyl-9-deacetyl-4-demethoxydaunorubicin (**1f**), originally developed by a research group at Roche,[8] because of their prominent anticancer activity, comparable with that of **1c**, **d**.

Numerous synthetic routes to the aglycones (**2c**, **d**) of **1c**, **d** have been explored.[3] However, the number of methods applicable to the preparation of the aglycones (**2e**, **f**) of **1e**, **f**,[8,9] seems to be quite limited in spite of their promising anticancer activity.[8a] While **2e**, **f** were first synthesized by employing the Diels–Alder reaction as a key synthetic strategy,[8] another synthesis of **2e** starting from D-lactose[9] has recently been reported. The latter synthetic scheme[9] seems to be superior to the original one[8] except for the non-stereoselective construction of the C_7-hydroxy group (the anthracycline numbering).

It was previously reported from these laboratories that (*R*)-2,5,12-trihydroxy-6,11-dioxo-1,2,3,4-tetrahydronaphthacene-2-carboxylic acid ((*R*)-**3**) could be directly converted to (*R*)-7-deoxy-4-demethoxydaunomycinone ((*R*)-**4**), the key intermediate to **2c**, **d**, through Grignard addition to the acyl imidazole derivative.[10] Since a large amount of optically pure (*R*)-**3** is available by conventional optical resolution of the readily obtainable racemic acid (($\pm$)-**3**),[10] we attempted to develop a novel synthetic route to **2e**, **f**, more practical than those so far reported,[8,9] by converting (*R*)-**3** to the corresponding primary alcohol ((*R*)-**11**).

1 : R² = (sugar)
2 : R² = H

	R¹	R³
a	OMe	$COCH_2OH$
b	OMe	COMe
c	H	$COCH_2OH$
d	H	COMe
e	H	CH_2OH
f	H	$CH_2OCONHPh$

	R
(±)- or (*R*)-3 :	OH
(±)- or (*R*)-4 :	Me
(±)- or (*R*)-5 :	OMe

	R¹	R²
(±)-6 :	H	H
(±)-8 :	Ac	Ac

(±)-7

(±)-9

(±)-10

	R¹	R²	R³
(±)- or (*R*)-11 :	H	H	H
(±)- or (*R*)-12 :	H	CMe_2	
(±)- or (*R*)-13 :	H	H	$SiMe_2$*tert*-Bu
(+)-14 :	OH	H	$SiMe_2$*tert*-Bu

Chart 1

This report deals with chemoselective reduction of the methyl ester ((*R*)-**5**) derived from (*R*)-**3** and elaboration of ((*R*)-**11**) to **2e**, **f** in a straightforward manner by sequential stereoselective $C_{7\alpha}$-hydroxylation (the anthracycline numbering) and urethane formation.[1]

Results and Discussion

At the outset, it was expected that chemoselective conversion of (*R*)-**3** to (*R*)-**11** could be simply accomplished by usual reduction of (*R*)-**5** with lithium aluminum hydride because the anthraquinone functionality present in the BCD ring system should be protected from hydride attack by intramolecular chelation with the adjacent aluminum phenoxide. However, this was not the case.

In order to save optically pure (*R*)-**5**, examinations to find the optimum reaction conditions were performed with the racemic ester ((±)-**5**). Thus, when (±)-**5** was treated with lithium aluminum hydride in tetrahydrofuran, both the ester and anthraquinone systems were simultaneously reduced, giving (±)-2-hydroxy-2-hydroxymethyl-5,12-dioxo-1,2,3,4-tetrahydronaphthacene ((±)-**6**) in 68% yield. The structure of (±)-**6** was rigorously determined by comparing its spectral data with those reported for (±)-2-acetyl-2-hydroxy-5,12-dioxo-1,2,3,4-tetrahydronaphthacene ((±)-**7**)[11] and by successful transformation to (±)-**11** (*vide infra*). Formation of (±)-**6** can be rationalized in terms of the reduction of two carbonyl groups of the anthraquinone moiety to the corresponding aluminum alkoxides followed by

concomitant dehydration and quinone formation during acidic work-up.

Aiming to confirm the structure of (±)-**6** and to explore the synthetic route to (±)-**11**, we examined the conversion of (±)-**6** to (±)-**11** through a reaction sequence similar to that employed for the synthesis of (±)-**4** from (±)-**7**.[11] Usual acetylation of (±)-**6** followed by reductive acetylation of the diacetate ((±)-**8**) with zinc produced the tetraacetate ((±)-**9**). This was derived to (±)-**11** by way of the anthraquinone tetraacetate ((±)-**10**) by successive oxidation and alkaline hydrolysis. Since (±)-**11** showed extremely low solubility in usual organic solvents, it was directly subjected to acetonide formation with 2,2-dimethoxypropane in the presence of *dl*-camphorsulfonic acid. The acetonide ((±)-**12**) having improved solubility could be readily purified by column chromatography. The overall yield of (±)-**12** from (±)-**6** was 34%.

Chemoselective reduction of (±)-**5** was further attempted by the use of diisobutylaluminum hydride or sodium bis(2-methoxyethoxy)aluminum hydride in toluene. However, these reactions simply produced complex mixtures of products in which (±)-**11** could not be detected by thin layer chromatography (TLC) analysis. Prior to the reductions of (±)-**5** described above, (±)-**3** was directly subjected to reduction with diborane or with sodium borohydride by way of the corresponding mixed anhydride.[12] However, these attempts were all fruitless.

Finally, reduction with lithium tri-*tert*-butoxyaluminum hydride[13] in dimethyl sulfoxide was found to be quite promising. Treatment of (±)-**5** with lithium tri-*tert*-butoxyaluminum hydride in dimethyl sulfoxide at room temperature for 5 h, followed by acetonide formation of crude (±)-**11**, afforded (±)-**12** in 55% overall yield. When crude (±)-**11** was silylated with 4-*tert*-butyldimethylsilyloxy-3-pentene-2-one in the presence of *p*-toluenesulfonic acid,[14] a 51% yield of the silyl ether ((±)-**13**) could be obtained after purification by column chromatography. As both acetonide formation and silylation, employed to increase the solubility of (±)-**11**, proceeded quantitatively on the pure sample of (±)-**11**, the chemical yields of (±)-**12** and (±)-**13** should reflect those of the reduction step. In this reduction, the starting ester ((±)-**5**) was always recovered in 19—34% yield and formation of a small amount of (±)-**6** was usually observed.[15]

Next, the established reaction conditions were applied to the reduction of optically pure (*R*)-**5**[16] derived from (*R*)-**3**.[10] Treatment of optically pure (*R*)-**5**, $[\alpha]_D^{20}$ $-60.0°$ (chloroform), by the same procedure as described for (±)-**5** produced (*R*)-**12**,[9b] $[\alpha]_D^{20}$ $-52.0°$ (chloroform), and (*R*)-**13**, $[\alpha]_D^{20}$ $-40.7°$ (chloroform), in 53% and 55% overall yields, respectively, by way of crude (*R*)-**11**. Recovery of (*R*)-**5** and formation of (*R*)-**6** were also observed by TLC analysis of the crude reduction product. The racemic and optically active alcohols ((±)- and (*R*)-**11**), $[\alpha]_D^{20}$ $-52.0°$ (chloroform), were regenerated from (±)-**12** and (*R*)-**13**, respectively, by treatment with concentrated hydrochloric acid. Taking into account the increased solubility, the $C_{7\alpha}$-hydroxylation (the anthracycline numbering) was directly attempted using (*R*)-**13** without deprotection.

According to the reported procedure,[17] bromination of (*R*)-**13** was attempted with bromine in carbon tetrachloride, and the bromide formed was treated with aqueous alkaline solution, giving the $C_{7\alpha}$-hydroxylated silyl ether ((+)-**14**) in 43% overall yield. While a small amount of the starting material ((*R*)-**13**) was recovered, formation of the undesired $C_{7\beta}$-epimer could not be detected in the nuclear magnetic resonance (NMR) spectrum of the crude reaction product. Similarly to the case of preparation of **2d** from (*R*)-**4**, highly stereoselective formation of (+)-**14** can be rationalized in terms of attack of the hydroxide anion hydrogen-bonded with the $C_{9\alpha}$-hydroxyl group.[16] Deprotection of (+)-**14** with aqueous hydrofluoric acid in acetonitrile afforded **2e**, $[\alpha]_D^{20}$ $+167°$ (dioxane), in a quantitative yield. The spectral data of this sample were almost identical with those reported.[9b]

Reaction of **2e** with phenyl isocyanate in pyridine at room temperature effected

chemoselective urethane formation to give **2f**, $[\alpha]_D^{20}$ +121° (dioxane), in 52% yield. Various urethanes useful as the aglycones of 9-carbamoyloxymethyl-9-deacetyl-4-demethoxydaunorubicins should be obtainable by the use of isocyanates other than phenyl isocyanates.

As described above, we have succeeded in developing a novel synthetic route to **2e, f**. Due to its brevity and directness, this approach is expected to be applicable to the practical synthesis of these unnatural anthracyclinones.

Experimental[18]

(±)- and (*R*)-Methyl 2,5,12-Trihydroxy-6,11-dioxo-1,2,3,4-tetrahydronaphthacene-2-carboxylate ((±)- and (*R*)-5)——The racemic and the optically pure esters ((±)- and (*R*)-**5**) showing mp 207.5—209.5 °C and mp 213—214 °C, $[\alpha]_D^{20}$ −60.0° (c=0.110, $CHCl_3$),[16] respectively (lit.,[10] mp 211.5—213.5 °C (for (±)-**5**) and mp 210.5—211.5 °C, $[\alpha]_D^{20}$ −60.0° (c=0.10, $CHCl_3$) (for (*R*)-**5**)), were prepared according to the reported method.[1] The NMR spectra of these samples were identical with those reported.[10]

(±)-2-Hydroxy-2-hydroxymethyl-1,2,3,4-tetrahydro-5,12-naphthacenedione ((±)-6)——A suspension of lithium aluminum hydride (264 mg, 7.0 mmol) in THF (20 ml) was added to a solution of (±)-**5** (508 mg, 1.4 mmol) in THF (50 ml) with stirring at room temperature. The mixture was stirred at the same temperature for 16 h, then heated at reflux for 1 h. Further lithium aluminum hydride (110 mg, 2.9 mmol) was added to the reaction mixture, and stirring under reflux was continued for 2 h. After being cooled in an ice bath, the mixture was diluted with saturated aqueous oxalic acid solution (50 ml), stirred for 0.5 h, then extracted with CH_2Cl_2. The combined organic extracts were washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, EtOAc) to afford (±)-**6** as a dark orange solid (288 mg, 68%). A sample recrystallized from EtOAc showed mp 234—237.5 °C. IR (KBr): 3450, 1655, 1615, 1590, 1400, 1295 cm^{-1}. NMR ($CDCl_3$) δ: 1.4—2.3 (4H, C_3-H_2 and OH×2), 2.6—3.1 (4H, m, C_1-H_2 and C_4-H_2), 3.63, 3.71 (2H, two d, J=each 12 Hz, CH_2O), 7.6—7.89 (2H, m, aromatic protons), 7.9—8.2 (2H, m, aromatic protons), 8.60 (2H, two s, C_6-H and C_{11}-H). MS m/z: 309 ($[M+1]^+$), 308 (M^+), 277 ($[M-CH_2OH]^+$).

(±)- and (*R*)-3,4-Dihydro-5,12-dihydroxy-2′,2′-dimethyl-spiro[1*H*-naphthaceno-2,4′-dioxolan]-6,11-dione ((±)- and (*R*)-12)——a) Preparation of (±)-**12** from (±)-**6**: A mixture of (±)-**6** (65.4 mg, 0.21 mmol), acetic anhydride (0.5 ml, 5.3 mmol), and DMAP (6.3 mg, 0.052 mmol) in pyridine (0.5 ml) was stirred at room temperature for 2 h, then concentrated *in vacuo*. The residue was dissolved in EtOAc, and this solution was washed successively with 3 M HCl, H_2O, and saturated NaCl. Drying over anhydrous $MgSO_4$, filtration and concentration *in vacuo* gave crude (±)-2-acetoxy-2-acetoxymethyl-1,2,3,4-tetrahydronaphthacene-5,12-dione ((±)-**8**) as a yellow solid (82.5 mg, 99%). NMR ($CDCl_3$) δ: 2.00, 2.14 (6H, two s, CH_3CO×2), 1.7—3.5 (6H, m, C_1-H_2, C_3-H_2, and C_{11}-H_2), 4.50, 4.66 (2H, two d, J= each 12 Hz, CH_2O), 7.5—7.8 (2H, m, aromatic protons), 7.9—8.1 (2H, m, aromatic protons), 8.57 (2H, two s, C_6-H and C_{11}-H). This was immediately subjected to the next reduction.

Zinc powder (58.5 mg, 0.89 mmol) was added to a mixture of crude (±)-**8** (82.5 mg, 0.21 mmol) and triethylamine (0.5 ml, 3.6 mmol) in acetic anhydride (2 ml), and the mixture was stirred at room temperature for 1 h. After filtration, the filtrate was concentrated *in vacuo*, and the residue was dissolved in EtOAc. The ethyl acetate solution was washed successively with 3 M HCl, H_2O, and saturated NaCl, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*, giving crude (±)-2,5,12-triacetoxy-2-acetoxymethyl-1,2,3,4-tetrahydronaphthacene ((±)-**9**) as a pale yellow solid (109 mg, 100%). NMR ($CDCl_3$) δ: 1.7—3.0 (6H, m, C_1-H_2, C_3-H_2, and C_4-H_2), 2.07, 2.44, 2.45, 2.48 (12H, four s, CH_3CO×4), 4.06 (2H, s, CH_2O), 7.3—7.5 (2H, m, aromatic protons), 7.8—8.1 (2H, m, aromatic protons), 8.23 (2H, s, C_6-H and C_{11}-H).

Chromium trioxide (88 mg, 0.88 mmol) was added to a mixture of (±)-**9** (109 mg, 0.23 mmol) in 80% aqueous AcOH with stirring at 50 °C. The stirring was continued for 3 h while two further lots of chromium trioxide (87 and 41 mg, total 216 mg, 2.2 mmol) were added at intervals of 1 h. The mixture was diluted with EtOAc, and washed successively with H_2O and saturated NaCl. Drying over anhydrous $MgSO_4$, filtration and concentration *in vacuo* gave crude (±)-2,5,12-triacetoxy-2-acetoxymethyl-1,2,3,4-tetrahydronaphthacene-6,11-dione ((±)-**10**) as a solid (93.9 mg, 81%). NMR ($CDCl_3$) δ: 1.7—3.6 (6H, m, C_1-H_2, C_3-H_2, and C_4-H_2), 1.93, 2.11, 2.51, 2.53 (12H, four s, CH_3CO×4), 4.56 (2H, s, CH_2O), 7.6—7.8 (2H, m, aromatic protons), 8.0—8.3 (2H, m, aromatic protons). This was directly subjected to hydrolysis and acetalizaion.

A 2 M aqueous NaOH solution (2 ml) was added to a solution of crude (±)-**10** (93.9 mg, 0.18 mmol) in THF (2 ml), and the mixture was stirred at room temperature for 15 min. After being acidified with 3 M HCl, the mixture was concentrated *in vacuo* to remove THF. Crude (±)-**11** separated as a red solid was collected by filtration (52.4 mg, 83%). This was subjected to acetonide formation by the same procedure as described in b), giving (±)-**12** as a reddish orange solid (27.5 mg, 34% overall from (±)-**6**) after purification by column chromatography. The NMR spectrum of this sample was identical with that of (*R*)-**6** obtained in d).

b) Preparation of (±)-**12** from (±)-**11**: 2,2-Dimethoxypropane (0.1 ml, 0.81 mmol) and CSA (3.8 mg,

0.016 mmol) were added to a solution of (±)-**11** (22.3 mg, 0.066 mmol) in THF (5 ml), and the mixture was stirred at room temperature for 2 h under an argon atmosphere. Further amounts of 2,2-dimethoxypropane (0.1 ml, 0.81 mmol) and CSA (8.0 mg, 0.034 mmol) were added to the reaction mixture, and stirring was continued for 3 h. The mixture was diluted with saturated $NaHCO_3$, and extracted with CH_2Cl_2. The combined organic extracts were washed with H_2O, dried over anhydrous $MgSO_4$, filtered, then concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, $CHCl_3$) to give (±)-**12** as reddish orange crystals (24.6 mg, 99%). Recrystallization from C_6H_6 gave a pure sample of (±)-**12** as reddish orange crystals, mp 263—265.5 °C. IR (KBr): 3475, 1625, 1590, 1410, 1370, 1265, 1245, 800 cm^{-1}. The NMR spectrum and MS of this sample were identical with those of (*R*)-**12** obtained in d).

c) Preparation of (±)-**12** from (±)-**5** by Way of (±)-**11**: Lithium tri-*tert*-butoxyaluminum hydride[13] (708 mg, 2.9 mmol) was added to a solution of (±)-**5** (68.2 mg, 0.19 mmol) in DMSO (5 ml), and the mixture was stirred at room temperature for 5 h. After being cooled in an ice bath, the mixture was diluted with saturated aqueous oxalic acid solution (10 ml), and extracted with a mixture of THF and EtOAc. The extracts were combined, washed with H_2O, dried over anhydrous $MgSO_4$, filtered, then concentrated *in vacuo*. The TLC analysis (SiO_2, C_6H_6–AcOEt 1 : 1) revealed that the residue contained (±)-**5** and (±)-**6** (a trace amount) in addition to the desired (±)-**11**. Without separation, this mixture was subjected to acetonide formation by a procedure similar to that described in b). Separation of the crude product by column chromatography gave (±)-**5** as a red solid (22.9 mg, 34%), and (±)-**12** as a red solid (38.6 mg, 55% from (±)-**5**). Spectral comparisons showed that (±)-**5** was identical with an authentic sample.[10] The NMR spectrum of (±)-**12** was identical with that of (*R*)-**12** described in d).

d) Preparation of (*R*)-**12** from (*R*)-**5** by Way of (*R*)-**11**: Lithium tri-*tert*-butoxyaluminum hydride (563 mg, 2.2 mmol) was added to a solution of (*R*)-**5** (54.5 mg, 0.15 mmol) in DMSO (5 ml), and the mixture was stirred at room temperature for 10 h. Treatment of the reaction mixture as described in c) gave crude (*R*)-**11** contaminated with (*R*)-**5** and (*R*)-**6** (a trace amount), after concentration of the combined organic extracts *in vacuo*. Acetonide formation of crude (*R*)-**11** as described for (±)-**11**, followed by separation by column chromatography (SiO_2, $CHCl_3$–EtOAc 20 : 1), gave (*R*)-**12** as reddish orange crystals (29.9 mg, 53%). In this case, separation of (*R*)-**5** was not attempted. Recrystallization from C_6H_6 gave pure (*R*)-**12** as reddish orange crystals, mp 221.5—222.5 °C and $[\alpha]_D^{20}$ −52.0° (c=0.050, $CHCl_3$) (lit.,[9b] mp 232—234 °C and $[\alpha]_D^{20}$ −52° (c=0.03, $CHCl_3$)). IR (KBr): 1625, 1590, 1410, 1260, 1240 cm^{-1}. NMR ($CDCl_3$) δ: 1.44 (6H, two s, Me_2C), 1.5—2.3 (2H, m, C_3-H_2), 2.9—3.2 (4H, m, C_1-H_2 and C_4-H_2), 3.95 (2H, s, CH_2O), 7.8—8.0 (2H, m, aromatic protons), 8.3—8.5 (2H, m, aromatic protons), 13.49, 13.50 (2H, two s, OH × 2). MS m/z: 381 ($[M+1]^+$), 380 (M^+), 322 ($[M-Me_2CO]^+$), 305 ($[M-Me_2CO_2]^+$). These spectral data were almost identical with those reported.[9b] *Anal.* Calcd for $C_{22}H_{20}O_6$: C, 69.49; H, 5.30. Found: C, 69.43; H, 5.50.

(±)- and (*R*)-2-(*tert*-Butyldimethylsilyloxymethyl)-2,5,12-trihydroxy-1,2,3,4-tetrahydronaphthacene-6,11-dione ((±)- and (*R*)-13)——a) Preparation of (±)-**13** from (±)-**11**: 4-(*tert*-Butyldimethylsilyloxy)-3-pentene-2-one[14] (85.3 mg, 0.40 mmol) and a THF solution of TSA (0.052 M solution, 0.25 ml, 0.013 mmol) were added to a solution of (±)-**11** (30.6 mg, 0.090 mmol) in DMF (3 ml), and the mixture was stirred at room temperature for 10 min under an argon atmosphere. After being diluted with H_2O, the mixture was extracted with CH_2Cl_2 and AcOEt. The organic extracts were combined, washed with H_2O, dried over anhydrous $MgSO_4$, filtered, then concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, $CHCl_3$–EtOAc 20 : 1) to give (±)-**13** as a reddish orange solid (41.3 mg, 100%). Recrystallization from a mixture of C_6H_6 and C_6H_{14} gave pure (±)-**13** as reddish orange crystals, mp 146—148 °C, IR (KBr): 3600, 3475, 1620, 1590, 1410, 1250, 840 cm^{-1}. The NMR spectrum of this sample was identical with that of (*R*)-**13** described in c).

b) Preparation of (±)-**13** from (±)-**5** by Way of (±)-**11**: Reduction of (±)-**5** (301 mg, 0.82 mmol) with lithium tri-*tert*-butoxyaluminum hydride[13] (5.22 g, 20.5 mmol) in DMSO (30 ml) for 5 h by the same procedure as described for the preparation of (±)-**12** gave a mixture of crude (±)-**11** contaminated with (±)-**5** and (±)-**6** (a trace amount) after evaporation of the organic extracts. This mixture was silylated by the same method as described in a) to afford (±)-**13** as a red solid (190 mg, 51%) after separation by column chromatography. The NMR spectrum of this sample was identical with that of (*R*)-**13** obtained in c). Separation by column chromatography gave (±)-**5** (57 mg, 19%) which was identical with an authentic sample[10] on the basis of spectral comparisons.

c) Preparation of (*R*)-**13** from (*R*)-**5** by Way of (*R*)-**11**: A solution of (*R*)-**5** (253 mg, 0.69 mmol) in DMSO (20 ml) was added to a solution of lithium tri-*tert*-butoxyaluminum hydride[13] (4.31 g, 17.0 mmol) in DMSO (15 ml), and the mixture was stirred at room temperature for 5 h. After being cooled in an ice bath, the mixture was diluted with saturated aqueous oxalic acid solution (50 ml), and extracted with $CHCl_3$. The chloroform extracts were combined, washed with 1 M HCl and H_2O, and dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave crude (*R*)-**11** as a red solid. The TLC analysis (SiO_2, C_6H_6–AcOEt 1 : 1) revealed that this was contaminated with (*R*)-**5** and (*R*)-**6** (a trace amount). Crude (*R*)-**11** was subjected to silylation as described in a). Separation of the crude silylation product by column chromatography gave (*R*)-**5** as an orange solid (67 mg, 26%) and (*R*)-**13** as a red solid (171 mg, 55% from (*R*)-**5**). The NMR spectrum of (*R*)-**5** was identical with that of an authentic sample.[10] Recrystallization from CCl_4 gave an analytical sample of (*R*)-**13** as reddish orange crystals, mp 158.5—159.5 °C and $[\alpha]_D^{20}$ −40.7° (c=0.059, $CHCl_3$). IR (KBr): 3600, 3450, 1620, 1590, 1410, 1250, 840 cm^{-1}. NMR ($CDCl_3$) δ: 0.13 (6H, two s, Me_2Si), 0.96 (9H, three s, Me_3CSi), 1.5—2.2 (2H, m, C_3-H_2), 2.57 (1H, s, C_2-OH), 2.7—3.1 (4H, m, C_1-H_2 and C_4-

H_2), 3.64 (2H, s, CH_2O), 7.7—7.9 (2H, m, aromatic protons), 8.2—8.4 (2H, m, aromatic protons), 13.27 (2H, two s, OH×2). MS *m/z*: 455 ($[M+1]^+$), 454 (M^+), 397 ($[M-CMe_3]^+$), 349 ($[M-SiMe_2CMe_3]^+$). *Anal.* Calcd for $C_{25}H_{30}O_6Si$: C, 66.05; H, 6.65. Found: C, 66.09; H, 6.94.

(±)- and (*R*)-2,5,12-Trihydroxy-2-hydroxymethyl-1,2,3,4-tetrahydronaphthacene-6,11-dione ((±)- and (*R*)-11) ——a) Preparation of (±)-11 from (±)-12: Concentrated hydrochloric acid (1 ml) was added to a suspension of (±)-**12** (107 mg, 0.28 mmol) in THF (3 ml), and the mixture was stirred at 60 °C for 1 h. After being diluted with H_2O, the aqueous mixture was extracted with a mixture of THF and EtOAc. The organic extracts were combined, washed with H_2O, then dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* gave (±)-**11** as a red solid (94 mg, 98%). Recrystallization of this sample from a mixture of THF and C_6H_6 gave pure (±)-**11** as red crystals, mp 267—269 °C. IR (KBr): 3450, 1620, 1585, 1410, 1250 cm^{-1}. The NMR spectrum of this sample was identical with that of (*R*)-**11** described in b).

b) Preparation of (*R*)-**11** from (*R*)-**13**: Concentrated hydrochloric acid (0.5 ml) was added to a solution of (*R*)-**13** (71.0 mg, 0.16 mmol) in THF (2 ml), and the mixture was stirred at room temperature for 0.5 h. After being diluted with H_2O, the mixture was worked up by a procedure similar to that described in a), giving (*R*)-**11** as an orange solid (55.3 mg, 100%) after concentration of the organic extracts *in vacuo*. Recrystallization from a mixture of THF and C_6H_6 gave pure (*R*)-**11** as orange crystals, mp 264.5—266.5 °C and $[\alpha]_D^{20}$ −52.0° (*c*=0.050, dioxane) (lit.,[9b] mp 235—238 °C and $[\alpha]_D^{20}$ −32° (*c*=0.062, dioxane)). IR (KBr): 3425, 1620, 1590, 1420, 1405, 1280, 1250 cm^{-1}. NMR (DMSO-d_6) δ: 1.64—1.80 (2H, m, C_3-H_2), 2.68 (2H, s, C_1-H_2), 2.70—2.85 (2H, m, C_4-H_2), 3.36—3.45 (2H, m, CH_2O), 4.24 (1H, s, C_2-OH), 4.60 (1H, t, *J*=5 Hz, $CH_2O\underline{H}$), 7.88—7.94 (2H, m, aromatic protons), 8.17—8.23 (2H, m, aromatic protons), 13.29 (2H, two s, OH×2). MS *m/z*: 341 ($[M+1]^+$), 340 (M^+), 309 ($[M-CH_2OH]^+$), 291 ($[M-CH_2OH-H_2O]^+$). These spectral data were very similar to those reported.[9b] *Anal.* Calcd for $C_{19}H_{16}O_6 \cdot 1/10H_2O$: C, 66.70; H, 4.77. Found: C, 66.70; H, 4.88.

(2*S*,4*S*)-(+)-2-(*tert*-Butyldimethylsilyloxy)methyl-2,4,5,12-tetrahydroxy-1,2,3,4-tetrahydronaphthacene-6,11-dione ((+)-14)——A solution of bromine in CCl_4 (0.089 M solution, 4.0 ml, 0.36 mmol) was added to a solution of (+)-**13** (163 mg, 0.36 mmol) in CCl_4 (35 ml) over 15 min with stirring in an ice bath under an argon atmosphere and irradiation with a tungsten lamp. After 1 h and 1.5 h, further amounts of a solution of bromine in CCl_4 (each 0.5 ml; total 5.0 ml, 0.45 mmol) were added, and the reaction was continued for 2 h. The mixture was diluted with 0.3 M NaOH (15 ml) in an ice bath, and stirred at the same temperature for 15 min. The alkaline mixture was cooled in an ice bath, diluted with H_2O, neutralized with 3 M HCl, then extracted with $CHCl_3$. The chloroform extracts were combined, washed with H_2O, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, $CHCl_3$, then $CHCl_3$–EtOAc 30:1), giving pure (+)-**14** as a reddish orange solid (72.5 mg, 43%), mp 138—147 °C $[\alpha]_D^{20}$ +135° (*c*=0.055, $CHCl_3$). A small amount of (+)-**13** (12.3 mg, 8%) was also recovered. IR (KBr): 3480, 1625, 1590, 1430, 1415, 1375, 1255, 1070, 840, 780 cm^{-1}. NMR ($CDCl_3$) δ: 0.13 (6H, two s, Me_2Si), 0.95 (9H, three s, Me_3CSi), 1.92 (1H, dd, *J*=15.0, 5.4 Hz, C_{3ax}-H), 2.29 (1H, ddd, *J*=15.0, 1.8, 1.7 Hz, C_{3eq}-H), 2.68 (1H, d, *J*=18.9 Hz, C_{1ax}-H), 3.13 (1H, dd, *J*=18.9, 1.7 Hz, C_{1eq}-H), 3.32 (1H, s, C_2-OH), 3.62, 3.64 (2H, two d, *J*=each 9.6 Hz, CH_2O), 3.90 (1H, d, *J*=7.0 Hz, C_4-OH), 5.28 (1H, ddd, *J*=7.0, 5.4, 1.8 Hz, C_4-H), 7.7–7.9 (2H, m, aromatic protons), 8.2—8.4 (2H, m, aromatic protons), 13.35 and 13.61 (2H, two s, C_5-OH and C_{12}-OH or *vice versa*). MS *m/z*: 471 ($[M+1]^+$), 470 (M^+), 434 ($[M-2H_2O]^+$), 395 ($[M-2H_2O-Me_3C]^+$), 377 ($[M-SiMe_2CMe_3]^+$).

(2*S*,4*S*)-(+)-2,4,5,12-Tetrahydroxy-2-hydroxymethyl-1,2,3,4-tetrahydronaphthacene-6,11-dione ((+)-2e)——An MeCN solution of hydrogen fluoride (0.68 M solution, 1 ml, 0.68 mmol) was added to a solution of (+)-**14** (66.1 mg, 0.14 mmol) in MeCN (5 ml), and the mixture was stirred at room temperature for 0.5 h. After being diluted with H_2O, the mixture was extracted with a mixture of THF and EtOAc. The organic extracts were combined, washed with H_2O, dried over anhydrous $MgSO_4$, filtered, then concentrated *in vacuo*, giving (+)-**2e** as a reddish orange solid (52.2 mg, 100%). Recrystallization from C_6H_6 gave pure (+)-**2e** as reddish orange crystals, mp 201.5—204.5 °C, $[\alpha]_D^{20}$ +167° (*c*=0.024, dioxane) and $[\alpha]_D^{30}$ +111° (*c*=0.052, THF) (lit.,[8a] $[\alpha]_D^{20}$ +131.3° (*c*=0.1, dioxane); lit.,[9b] mp 230 °C, $[\alpha]_D$ +95° (*c*=0.05, THF)).[19] IR (KBr): 3460, 1625, 1590, 1415, 1405, 1375, 1265, 1240, 1050, 985 cm^{-1}. NMR (pyridine-d_5) δ: 2.31 (1H, dd, *J*=14.2, 4.4 Hz, C_{3ax}-H), 2.72 (1H, ddd, *J*=14.2, 2.1, 1.8 Hz, C_{3eq}-H), 3.24 (1H, d, *J*=18.9 Hz, C_{1ax}-H), 3.68 (1H, dd, *J*=18.9, 1.8 Hz, C_{1eq}-H), 4.08, 4.10 (2H, d, *J*=each 10.8 Hz, CH_2O), 5.69 (1H, dd, *J*=4.4, 2.1 Hz, C_4-H), 6.49 (3H, br s, C_2-OH, C_4-OH, and CH_2OH), 7.70—7.77 (2H, m, aromatic protons), 8.33—8.40 (2H, m, aromatic protons), 13.79, 14.08 (2H, two s, C_5-OH and C_{12}-OH or *vice versa*). MS *m/z*: 357 ($[M+1]^+$), 356 (M^+), 338 ($[M-H_2O]^+$), 320 ($[M-2H_2O]^+$), 308 ($[M-H_2O-CH_2OH+1]^+$), 307 ($[M-H_2O-CH_2OH]^+$). These spectral data were almost identical with those reported.[9b] *Anal.* Calcd for $C_{19}H_{16}O_7 \cdot 1/4H_2O$: C, 63.24; H, 4.61. Found: C, 63.43; H, 4.55.

(2*S*,4*S*)-(+)-2,4,5,12-Tetrahydroxy-2-phenylcarbamoyloxymethyl-1,2,3,4-tetrahydronaphthacene-6,11-dione ((+)-2f)——A pyridine solution of phenyl isocyanate (0.19 M solution, 0.44 ml, 0.082 mmol) was added to a solution of (+)-**2e** (29.0 mg, 0.081 mmol) in pyridine (1.5 ml), and the mixture was stirred at room temperature for 3 h. After further amounts of a pyridine solution of phenyl isocyanate (0.19 M solution) were added three times to the reaction mixture every 2 h (0.22 ml×2, then 0.11 ml×1, total 0.99 ml, 0.19 mmol), the stirring was continued at room temperature for 5 h. After concentration *in vacuo*, the residue was dissolved in EtOAc, and the insoluble materials

were removed by filtration. The filtrate was washed successively with 3 M HCl and H_2O, dried over anhydrous $MgSO_4$, filtered, then concentrated *in vacuo*. The residue was twice purified by column chromatography (SiO_2, C_6H_6–EtOAc 1 : 1) to afford (+)-**2f** as an orange solid (20.3 mg, 52%). An analytical sample was obtained as orange crystals by recrystallization from Et_2O–C_6H_{14}, mp 222—223 °C and $[\alpha]_D^{20}$ +121° (c=0.053, dioxane), (lit.,[8b] mp 225—226 °C and $[\alpha]_D^{20}$ +136.0° (c=0.05, dioxane)).[19] IR (KBr): 3450, 1735, 1715, 1625, 1590, 1440, 1235 cm^{-1}. NMR ($CDCl_3$) δ: 1.97 (1H, dd, J=15.0, 5.4 Hz, C_{3ax}-H), 2.46 (1H, ddd, J=15.0, 2.0, 1.7 Hz, C_{3eq}-H), 2.73 (1H, d, J=18.9 Hz, C_{1ax}-H), 3.30 (1H, dd, J=18.9, 1.7 Hz, C_{1eq}-H), 3.69 (1H, d, J=4.6 Hz, C_4-OH), 3.96 (1H, s, C_2-OH), 4.33 (2H, s, CH_2O), 5.36 (1H, ddd, J=5.4, 4.6, 2.0 Hz, C_1-H), 6.88 (1H, brs, NH), 7.0—7.5 (2H, m, aromatic protons), 7.75—8.0 (2H, m, aromatic protons), 13.32, 13.58 (2H, two s, C_5-OH and C_{12}-OH, or *vice versa*). MS m/z: 476 ($[M+1]^+$), 475 (M^+), 356 ($[M-H_2O-1]^+$), 338 ($[M-2H_2O+1]^+$), 320 ($[M-H_2O-C_6H_5NHCOO-1]^+$). *Anal.* Calcd for $C_{26}H_{21}NO_8 \cdot 1/3H_2O$: C, 64.86; H, 4.54; N, 2.91. Found: C, 64.91; H, 4.43; N, 2.84.

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16) The optical purity of (*R*)-**5** could be readily estimated by measuring the NMR spectrum of the dimethyl ether of (*R*)-**5** in the presence of a chiral shift reagent. See ref. 10.
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18) All melting points were determined with a Yamato MP-21 melting point apparatus and are uncorrected. Infrared (IR) spectral measurements were performed on a JASCO A-202 diffraction grating infrared spectrometer. NMR spectra were measured with a Varian EM-390 spectrometer (90 MHz), a Hitachi R-90H spectrometer (90 MHz), and a Bruker AM-400 spectrometer (400 MHz). All signals were expressed as ppm downfield from tetramethylsilane (TMS) used as an internal standard (δ-value). Mass spectra (MS) were taken with a Hitachi RMU-6MG mass spectrometer. Measurements of optical rotations were carried out with a

Horiba SEPA-200 automatic digital polarimeter. Wakogel C-200 was used as an adsorbent for column chromatography. All reactions and measurements were carried out in anhydrous solvents. In particular, tetrahydrofuran, ether, and dioxane were freshly distilled from sodium benzophenone ketyl, and dichloromethane was freshly distilled from calcium hydride. The following abbreviations are used for reagents and solvents: acetic acid (AcOH), acetone (Me_2CO), acetonitrile (MeCN), benzene (C_6H_6), *dl*-camphorsulfonic acid (CSA), carbon tetrachloride (CCl_4), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), 4-dimethylaminopyridine (DMAP), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ether (Et_2O), ethyl acetate (EtOAc), hexane (C_6H_{14}), tetrahydrofuran (THF), *p*-toluenesulfonic acid (TSA).

19) Because of the extremely low solubility and characteristic reddish orange color of the sample, measurement of optical rotation was carried out at a low concentration. This might be the reason why the observed rotation value is fairly different from the reported value.

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Studies on Tetrahydroisoquinolines. XXIX.[1] Reaction of 7-Acetoxy-1,2,3,4,6,7-hexahydro-1-(2-(3′,4′-dimethoxy- or 3′,4′-methylenedioxyphenyl)ethyl)-7-methoxy-2-methyl-6-oxo-isoquinoline (*o*-Quinol Acetate) with Acetic Anhydride in the Presence of Acid

OSAMU HOSHINO,[a] KYOKO KIKUCHI,[a] HIDETO OGOSE,[a] BUNSUKE UMEZAWA,*,[a] and YOICHI IITAKA[b]

Faculty of Pharmaceutical Sciences, Science University of Tokyo,[a] 12, Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan and Faculty of Pharmaceutical Sciences, University of Tokyo,[b] 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

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Treatment of *o*-quinol acetates (**5a**, **b**) of 1,2,3,4-tetrahydro-1-phenethylisoquinolin-6-ols (**6a**, **b**) with acetic anhydride in the presence of an acid (concentrated H_2SO_4, $BF_3 \cdot Et_2O$ or CF_3COOH) gave 2-acetoxyhomoaporphines (**4a**, **b**) and/or aldehyde-amides (**7a**, **b**), and the ratio of the products was strongly dependent on the choice of the acid and the solvent. A mechanistic pathway is proposed.

Keywords——lead tetraacetate oxidation; X-ray analysis; *o*-quinol acetate; 1,2,3,4-tetrahydro-1-phenethylisoquinolin-6-ol; aldehyde-amide; 2-acetoxyhomoaporphine; acetic anhydride; concentrated sulfuric acid; boron trifluoride etherate; trifluoroacetic acid

Previously, we have reported that reaction of *o*-quinol acetates (*o*-QAs) (**1**) with acetic anhydride (Ac_2O) containing concentrated sulfuric acid (conc. H_2SO_4) or with Ac_2O–conc. H_2SO_4 in acetonitrile (CH_3CN) gives rise to 2-acetoxyaporphines (**2**)[2] or 1,2-diacetoxyaporphines (**3**).[3] In order to extend the methodology to the synthesis of 2-acetoxyhomoaporphines (**4a**, **b**), which are of interest pharmacologically, reaction of *o*-QAs (**5a**, **b**) derived from 1,2,3,4-tetrahydro-1-phenethylisoquinolin-6-ols (**6a**, **b**) was explored. The present paper is concerned with the formation of 2-acetoxyhomoaporphines (**4a**, **b**) and/or aldehyde-amides (**7a**, **b**) from *o*-QAs (**5a**, **b**) by treatment with Ac_2O containing an acid.

The starting material (**6a**) was prepared as follows. Heating of 2-(3-benzyloxy-4-methoxyphenyl)ethylamine[4] and 3-(3,4-dimethoxyphenyl)propionic acid[5] at 160 °C for 6 h gave an amide (**8a**),[6] Bischler–Napieralski reaction of which afforded 3,4-dihydroisoquinoline hydrochloride (**9a** · HCl)[6] in 68% overall yield (based on the amine oxalate). Reduction of **9a** with sodium borohydride ($NaBH_4$) followed by *N*-methylation gave 6-benzyloxy-1,2,3,4-tetrahydro-2-methylisoquinoline (**10a**), which was debenzylated by catalytic hydrogenolysis over palladium on carbon to give 1,2,3,4-tetrahydroisoquinolin-6-ol (**6a**) in 68.4% overall yield. Analogously, **6b** was prepared starting from the phenethylamine and 3-(3,4-methylenedioxyphenyl)propionic acid.[7]

As reported previously,[2] oxidation of **6a**, **b** with lead tetraacetate in methylene chloride (CH_2Cl_2) gave quantitatively 1 : 1 diastereomeric mixtures of *o*-QAs (**5a**, **b**), which rapidly decomposed on standing at room temperature to leave complex mixtures containing 4-acetoxytetrahydroisoquinolin-6-ols (**11a**, **b**). The spectral data for **5a**, **b** are shown in Table I.

Treatment of *o*-QA (**5a**) with Ac_2O–conc. H_2SO_4 at room temperature for 30 min gave

1 : n=1
5 : n=2

2 : n=1, R^1=Ac
4 : n=2, R^1=Ac
13 : n=2, R^1=H
14 : n=2, R^1=CH_3

3

a : R=CH_3 b : R+R=CH_2

6 : R^1=H
11 : R^1=OAc

7

Chart 1

8 9 10

a : R=CH_3 b : R+R=CH_2

Chart 2

oily products, which were subjected to preparative thin layer chromatography (TLC) to afford an aldehyde-amide (**7a**) (20.5%) and 2-acetoxyhomoaporphine (**4a**) (25.3%). The mass spectrum (MS) of the former (**7a**) indicated a molecular formula of $C_{25}H_{31}NO_7$ and the proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table II) showed the signals of a formyl group as a singlet (1H) at δ 9.59, the acetyl group of an acetamido grouping as a pair of singlets[8] (3H) at δ 1.67 and 1.99, and of an *N*-methyl group as a pair of singlets[8] (3H) at δ 2.66 and 2.71. The infrared (IR) spectrum (Table II) exhibited absorption bands due to phenolic acetoxyl, formyl, and amido groups at 1770, 1730, and 1635 cm^{-1}, respectively.

On the basis of these spectral data, the structure of the aldehyde-amide (**7a**) was deduced to be 4-acetoxy-2-(2-(*N*-acetyl-*N*-methylamino)ethyl)-5-methoxyphenyl-2′-(2-formylethyl)-4′,5′-dimethoxybenzene. Furthermore, the position of the acetoxyl group in **7a** was confirmed by the X-ray crystallographic analysis of a methoperchlorate (**12**) derived from **7a**. An ORTEP drawing of **12** is depicted in Fig. 1.

The ^{1}H-NMR spectrum (Table II) of the latter (**4a**) showed a singlet signal (3H) due to a methoxyl group at the 1-position at δ 3.28 and of three singlet signals (each 1H) due to aromatic protons at δ 6.75, 6.80, and 7.03. From these spectral data, microanalysis, and

TABLE I. Spectral Data for *o*-Quinol Acetates (**5a, b**)

o-QA	IR (cm^{-1})	^{1}H-NMR δ (100 MHz)		
		NCH_3[a]	OAc[a]	7-OCH_3[a]
5a	1745, 1690	2.38 (s)	2.08 (s)	3.43 (s)
		2.44 (s)	2.11 (s)	3.44 (s)
5b	1745, 1685	2.36 (s)	2.08 (s)	3.44 (s)
		2.43 (s)	2.12 (s)	3.45 (s)

a) A 1 : 1 ratio was observed. Abbreviation: s, singlet.

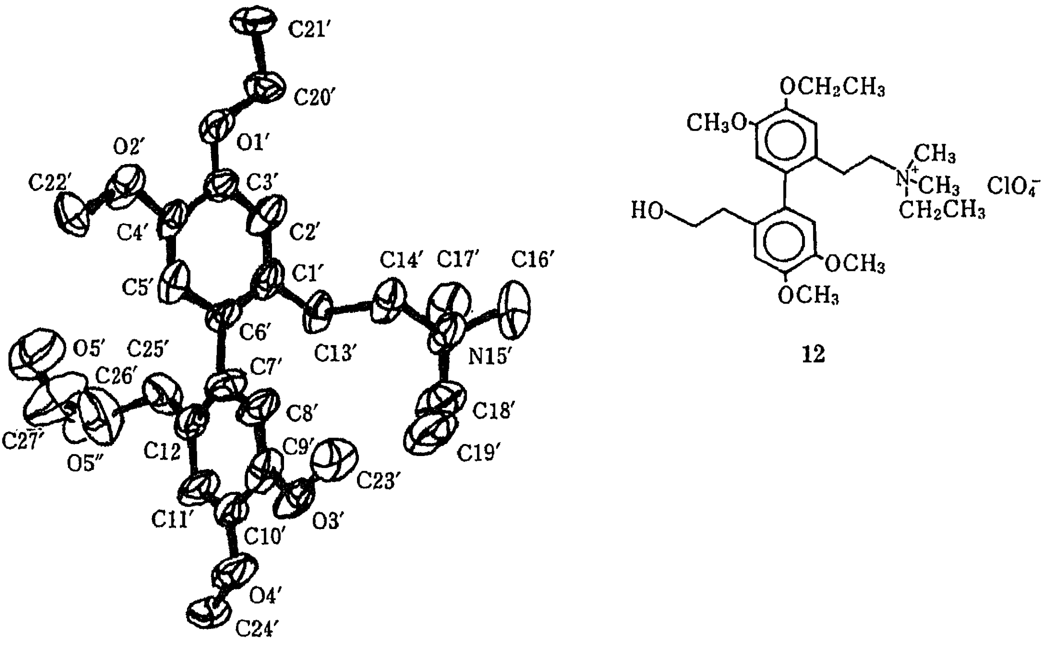

Fig. 1. An ORTEP Drawing of **12**

chemical conversion into a known compound 1,2,10,11-tetramethoxyhomoaporphine (**14a**),[6] the structure of **4a** was determined to be 2-acetoxy-1,10,11-trimethoxyhomoaporphine.

In contrast to the case of *o*-QAs (1),[2] the reaction of **5a** was proved to give 2-acetoxyhomoaporphine (**4a**) accompanied with aldehyde-amide (**7a**).

Although shortening of the reaction time did not change the ratio of products, employment of boron trifluoride etherate ($BF_3 \cdot Et_2O$) instead of conc. H_2SO_4 increased the yield of **4a**. With trifluoroacetic acid (TFA), however, the ratio of products dramatically changed to afford **7a** mainly. The results are summarized in Table III.

The pathway of formation of **4a** and **7a** may be as depicted in Chart 3. Namely, the ring closure of **5a** would occur through an intermediate A (7-*endo*-trigonal process)[9] and/or B (6-*exo*-trigonal process),[9] followed by rearrangement of the C6′–C8a to C6′–C8 bonds to give **4a**. On the other hand,[10] cleavage of the C1–C8a bond in B through a vinylogous retro-aldol type reaction would lead to an intermediate C. Further hydrolysis and acetylation of C would give rise to **7a**.[11]

The above mechanistic considerations strongly suggested that fixing the lone pair electrons on nitrogen would favor the formation of **4** over **7**. Therefore, preferential formation of **4** was expected to be possible by the use of conc. H_2SO_4 or $BF_3 \cdot Et_2O$ in aprotic

Table II. Spectral Data and Elemental Analysis of 2-Acetoxyhomoaporphines (**4a, b**) and Aldehyde-Amides (**7a, b**)

Products	IR (cm^{-1})	^{1}H-NMR δ (100 MHz)[a] NAc	NCH_3	OAc	OCH_3	OCH_2O	ArH	CHO	mp (°C) (Recrystn. solvent)	Formula	Analysis (%) Calcd (Found) C	H	N
4a	1760	—	2.39 (s)	2.35 (s)	3.23 (s) 3.87 (s) 3.94 (s)	—	6.75 (1H, s) 6.80 (1H, s) 7.03 (1H, s)	—	210.5— 211.5[b] (MeOH)	$C_{29}H_{29}N_4O_{12}$	55.68 (55.50	4.67 4.90	8.96 8.97)
7a	1770 1730 1635	1.67 (s) 1.99 (s) (1:1)	2.66 (s) 2.71 (s) (1:1)	2.34 (s)	3.79 (s) 3.80 (s) (1:1) 3.85 (s) 3.90 (s)	—	6.60—6.96 (4H, m)	9.59 (br s)	—[c]	$C_{25}H_{31}NO_7$	457.2098 (457.2090)[d]		
4b	1765	—	2.39 (s)	2.34 (s)	3.33 (s)	5.93 (1H, d) 5.96 (1H, d) (J=1)	6.72 (1H, s) 6.80 (1H, s) 6.95 (1H, s)	—	220— 220.5[b] (MeOH)	$C_{28}H_{25}N_4O_{12}$	55.18 (55.06	4.13 4.39	9.19 9.09)
7b	1775 1735 1640	1.77 (s) 2.00 (s) (1:1)	2.71 (s) 2.75 (s) (1:1)	2.33 (s)	3.78 (s) 3.79 (s) (1:1)	5.95 (2H, s)	6.56—6.96 (4H, m)	9.59 (br s)	—[c]	$C_{24}H_{27}NO_7$	441.1785 (441.1770)[d]		

a) Abbreviations are as follows: s, singlet; br s, broad singlet; d, doublet; m, multiplet. *b*) A picrate. *c*) An oil. *d*) High-resolution mass spectrum m/z (M^+).

Table III. Reaction Conditions and Yields of 2-Acetoxyhomoaporphines (**4a, b**) and Aldehyde-Amides (**7a, b**)

o-QA	Reaction conditions[a] Acid (ml)	Reaction time (min)	Yields (%) 4	7
5a	Conc. H_2SO_4 (0.3)	30	25.3	20.5
	Conc. H_2SO_4 (0.3)	10	24.3	10.7
	$BF_3 \cdot Et_2O$ (0.82)	60	55.6	14.8
	TFA[b] (0.5)	30	6.0	44.8
5b	Conc. H_2SO_4 (0.3)	10	26.9	20.5
	Conc. H_2SO_4 (0.3)[c]	60	62.0	5.2
	TFA[b] (0.5)	30	4.2	49.5

a) *o*-QAs (**5**) prepared from **6** (100 mg) (see Experimental) and Ac_2O (1 ml) were used. *b*) TFA: CF_3COOH. *c*) CH_3CN (50 ml) was used.

Chart 3

Chart 4

Table IV. Reaction Conditions and Yields of 2-Acetoxyhomoaporphine (**4a**) and Aldehyde-Amide (**7a**)

Entry	Reaction conditions[a]			Yield (%)	
	Solvent (ml)	Acid (ml)	Reaction time (min)	**4a**	**7a**
1	CH_2Cl_2 (50)	Conc. H_2SO_4 (0.3)	60	53.8	—
2	CH_3CN (50)	Conc. H_2SO_4 (0.3)	60	69.8	10.2
3	CH_3NO_2 (50)	Conc. H_2SO_4 (0.3)	10	80.2	—
4	CH_3NO_2 (10)	Conc. H_2SO_4 (0.3)	60	62.3	—
5	CH_3CN (50)	$BF_3 \cdot Et_2O$ (0.82)	60	61.0	12.3
6	CH_3NO_2 (50)	$BF_3 \cdot Et_2O$ (0.82)	60	78.6	—
7	CH_2Cl_2 (30)	TFA[b] (0.5)	30	—	50.5
8	CH_3CN (50)	TFA[b] (0.5)	60	—	29.3

a) *o*-QA (**5a**) prepared from **6a** (100 mg) (see Experimental) and Ac_2O (1 ml) were used. *b*) TFA: CF_3COOH.

polar solvents, which would be effective to fix the lone pair electrons on nitrogen. Indeed, treatment of **5a** with Ac_2O–conc. H_2SO_4 in CH_2Cl_2 gave only **4a** in 53.8% yield. Although employment of CH_3CN increased the yield of **4a**, the formation of **7a** was also observed (entry

2). With nitromethane (CH_3NO_2), however, **4a** was obtained in a good yield (entry 3). Furthermore, with $Ac_2O-BF_3 \cdot Et_2O$ in CH_3CN or CH_3NO_2 a similar trend was observed (entries 5 and 6). On the other hand, with Ac_2O–TFA in CH_2Cl_2 or CH_3CN, **7a** was formed as a sole product (entries 7 and 8). The results are listed in Table IV. Analogously, treatment of **5b** with Ac_2O containing acid was carried out to afford **4b** and **7b**, respectively (Table III). Acidic hydrolysis of **4a, b** gave 2-hydroxyhomoaporphines (**13a, b**) in good yields.

In conclusion, treatment of *o*-QAs (**5a, b**) with Ac_2O in the presence of an acid gave 2-acetoxyhomoaporphines (**4a, b**) and/or aldehyde-amides (**7a, b**). This is the first report of the formation of the latter products in the reaction of *o*-QAs with Ac_2O containing an acid.

Experimental

All melting points were measured on a Büchi melting point apparatus and are uncorrected. IR spectra were measured on a Hitachi 215 infrared spectrometer in $CHCl_3$ solution. ^1H-NMR spectra were taken with a JEOL-FX 100 spectrometer (100 MHz) in $CDCl_3$ solution using Me_4Si as an internal standard. MS were run on a Hitachi RMU-7M instrument at 70 eV. Preparative TLC was performed on Kieselgel HF_{254} (0.5 mm thick) (Merck) with $CHCl_3$–MeOH–AcOEt (8 : 1 : 1) as a developing solvent, unless otherwise noted.

***N*-2-(3-Benzyloxy-4-methoxyphenyl)ethyl-3-(3′,4′-dimethoxyphenyl)propionamide (8a)**——A mixture of 2-(3-benzyloxy-4-methoxyphenyl)ethylamine[4] (22.8 g), obtained from the oxalate (34.9 g), and 3-(3,4-dimethoxyphenyl)propionic acid[5] (23.7 g) was heated at 160 °C for 6 h. The product was taken up in $CHCl_3$ (250 ml). Usual work-up of the $CHCl_3$ solution gave a colorless solid (**8a**) (38.1 g, 84% based on the oxalate), mp 96—98 °C (benzene) (lit.[6] 88.3—89.8 °C). IR cm^{-1}: 3430 (NH), 1660 (CONH). ^1H-NMR δ: 3.69 (9H, s, $3 \times OCH_3$), 4.98 (2H, s, OCH_2Ar). *Anal.* Calcd for $C_{27}H_{31}NO_5$: C, 72.14; H, 6.95; N, 3.12. Found: C, 72.20; H, 6.75; N, 3.03.

***N*-2-(3-Benzyloxy-4-methoxyphenyl)ethyl-3-(3′,4′-methylenedioxyphenyl)propionamide (8b)**——A mixture of 2-(3-benzyloxy-4-methoxyphenyl)ethylamine[4] (4.0 g) and 3-(3,4-methylenedioxyphenyl)propionic acid[7] (3.7 g) was heated at 160 °C for 2.5 h. Usual work-up of the reaction mixture gave colorless crystals (**8b**) (6.0 g, 89.8%), mp 132.5—133.5 °C (benzene–hexane). IR cm^{-1}: 3430 (NH), 1665 (CONH). ^1H-NMR δ: 3.84 (3H, s, OCH_3), 5.06 (2H, s, OCH_2Ar), 5.86 (2H, s, OCH_2O). *Anal.* Calcd for $C_{26}H_{27}NO_5$: C, 72.04; H, 6.28; N, 3.23. Found: C, 72.27; H, 6.23; N, 3.18.

6-Benzyloxy-3,4-dihydro-7-methoxy-1-(2-(3′,4′-dimethoxyphenyl)ethyl)isoquinoline Hydrochloride (9a · HCl)——A solution of the amide (**8a**) (2.5 g) and $POCl_3$ (10 ml) in CH_2Cl_2 (25 ml) was refluxed for 3 h. Removal of the solvent *in vacuo* gave an oil, which was crystallized by trituration in a mixture of hexane, ether, and iso-PrOH to give pale yellow crystals (**9a** · HCl) (2.13 g, 82%), mp 188—189 °C (iso-PrOH) (lit.[6] 173.8—174.7 °C). *Anal.* Calcd for $C_{27}H_{30}NO_4 \cdot HCl$: C, 69.30; H, 6.64; N, 2.99. Found: C, 69.09; H, 6.49; N, 3.04.

6-Benzyloxy-3,4-dihydro-7-methoxy-1-(2-(3′,4′-methylenedioxyphenyl)ethyl)isoquinoline Hydrochloride (9b · HCl)——A solution of the amide (**8b**) (1.4 g) and $POCl_3$ (6 ml) in CH_2Cl_2 (15 ml) was refluxed for 3 h. Work-up of the reaction mixture as noted for **8a** gave pale yellow crystals (**9b** · HCl) (1.05 g, 72%), mp 198—199 °C (MeOH–iso-PrOH). IR cm^{-1}: 2700—2300 (br), 1660, 1575 (C=N). ^1H-NMR δ: 3.87 (3H, s, OCH_3), 5.24 (2H, s, OCH_2Ar), 5.88 (2H, s, OCH_2O), 7.38 (5H, s, C_6H_5). *Anal.* Calcd for $C_{26}H_{26}NO_4 \cdot HCl$: C, 67.74; H, 5.79; N, 3.04. Found: C, 67.59; H, 5.90; N, 3.04.

1,2,3,4-Tetrahydro-6-hydroxy-7-methoxy-1-(2-(3′,4′-dimethoxyphenyl)ethyl)-2-methylisoquinoline (6a)——$NaBH_4$ (1.4 g) was added portionwise to an ice-cooled, stirred solution of the free amine (**9a**) (22.2 g) in MeOH (240 ml) and the whole was stirred at room temperature for 2 h. Removal of the solvent *in vacuo* gave an oil. H_2O was added to the oil and the product was taken up in $CHCl_3$. Usual work-up of the $CHCl_3$ extract gave an oil (22 g, 99%). A solution of the crude oil (22 g) and 37% aqueous formaldehyde (20 g) in MeOH (220 ml) was stirred at room temperature for 2 h. $NaBH_4$ (9.6 g) was added portionwise to the ice-cooled, stirred mixture over a period of 2.5 h and stirring was continued at room temperature for 1 h. Usual work-up of the reaction mixture gave an oil (**10a**) (20.5 g, 90%). A solution of the crude **10a** (5.5 g), 2% aqueous $PdCl_2$ (14 ml), and active carbon (1.36 g) in MeOH (200 ml) was shaken with H_2 at room temperature for 1.4 h. After filtration to remove the catalyst, usual work-up of the reaction mixture gave pale yellow crystals (**6a**) (3.3 g, 76.7%) mp 113—114 °C (ether–hexane). IR cm^{-1}: 3550 (OH). ^1H-NMR δ: 2.46 (3H, s, NCH_3), 3.80, 3.83, 3.84 (each 3H, s, $3 \times OCH_3$), 6.47, 6.60 (each 1H, s, $2 \times ArH$), 6.64—6.82 (3H, m, $3 \times ArH$). High-resolution MS Calcd for $C_{21}H_{27}NO_4$ (M^+) *m/z*: 357.1937. Found: 357.1915.

1,2,3,4-Tetrahydro-6-hydroxy-7-methoxy-2-methyl-1-(2-(3′,4′-methylenedioxyphenyl)ethyl)isoquinoline (6b)——$NaBH_4$ (0.4 g) was added portionwise to an ice-cooled, stirred solution of the free amine (**9b**) (8.7 g) in MeOH (150 ml) and the whole was stirred at room temperature for 2 h. Usual work-up of the reaction mixture gave an oil (9.6 g), which was methylated as noted for **9a** to give an oil (**10b**) (9.0 g, 99%). A mixture of the crude **10b** (4.1 g), 2% aqueous $PdCl_2$ (12 ml), and active carbon (1.1 g) in MeOH (200 ml) was shaken with H_2 at room temperature for

TABLE V. Atomic Coordinates ($\times 10^4$) and Equivalent Isotropic Temperature Factors

No.	Atom	x	y	z	B_{eq} (Å^2)
1	Cl	7342 (4)	501 (3)	8936 (4)	8.2 (0.1)
2	O1(Cl)	6949 (20)	773 (11)	9864 (16)	24.5 (0.7)
3	O2(Cl)	8315 (14)	445 (15)	9000 (30)	29.3 (1.0)
4	O3(Cl)	6930 (17)	−143 (10)	8509 (19)	21.7 (0.6)
5	O4(Cl)	7224 (22)	1029 (11)	8277 (16)	22.7 (0.7)
6	Cl′	2508 (3)	2844 (2)	1375 (4)	7.5 (0.1)
7	O1(Cl′)	3002 (20)	3332 (12)	2160 (21)	24.2 (0.8)
8	O2(Cl′)	1963 (13)	2457 (10)	1948 (19)	18.1 (0.6)
9	O3(Cl′)	1940 (14)	3222 (15)	715 (21)	25.1 (0.8)
10	O4(Cl′)	3327 (12)	2487 (8)	889 (14)	14.2 (0.4)
11	C1	4677 (10)	2828 (7)	8044 (11)	4.6 (0.3)
12	C2	4785 (10)	3214 (7)	9152 (12)	5.1 (0.3)
13	C3	5496 (11)	3752 (7)	9356 (11)	4.9 (0.3)
14	C4	6131 (10)	3880 (7)	8504 (11)	4.5 (0.3)
15	C5	6046 (10)	3478 (7)	7448 (11)	4.7 (0.3)
16	C6	5326 (10)	2926 (7)	7232 (12)	4.9 (0.3)
17	C7	5312 (10)	2477 (7)	6095 (11)	4.5 (0.3)
18	C8	6119 (10)	1945 (6)	5928 (11)	4.3 (0.2)
19	C9	6185 (10)	1554 (7)	4832 (11)	4.7 (0.3)
20	C10	5466 (11)	1709 (7)	3937 (11)	5.2 (0.3)
21	C11	4687 (11)	2224 (7)	4125 (12)	5.3 (0.3)
22	C12	4578 (11)	2621 (7)	5221 (11)	5.0 (0.3)
23	C13	3876 (11)	2233 (7)	7855 (12)	5.0 (0.3)
24	C14	4338 (10)	1587 (7)	8429 (12)	5.0 (0.3)
25	N15	3665 (9)	916 (6)	8205 (9)	5.4 (0.2)
26	C16	2598 (11)	1120 (9)	8688 (15)	7.3 (0.4)
27	C17	4196 (13)	329 (8)	8843 (14)	7.3 (0.4)
28	C18	3441 (12)	656 (8)	6950 (13)	7.2 (0.3)
29	C19	4438 (14)	455 (9)	6352 (15)	8.4 (0.4)
30	C20	5008 (12)	4047 (9)	11262 (11)	6.5 (0.3)
31	C21	5416 (13)	4575 (9)	12286 (13)	7.5 (0.4)
32	C22	7389 (13)	4642 (9)	7923 (13)	7.3 (0.4)
33	C23	7648 (12)	848 (9)	5524 (13)	7.2 (0.3)
34	C24	5057 (16)	1569 (10)	1938 (12)	9.5 (0.4)
35	C25	3703 (12)	3202 (8)	5367 (14)	6.5 (0.3)
36	C26	4106 (15)	3913 (8)	5051 (17)	9.0 (0.4)
37	C27	3202 (20)	4455 (10)	5093 (26)	15.8 (0.8)
38	O1	5682 (7)	4162 (5)	10390 (7)	5.6 (0.2)
39	O2	6797 (7)	4448 (5)	8807 (8)	5.8 (0.2)
40	O3	6940 (7)	1027 (5)	4607 (8)	6.0 (0.2)
41	O4	5669 (8)	1329 (5)	2903 (8)	6.9 (0.2)
42	O5	3658 (17)	5053 (11)	4827 (15)	18.5 (0.6)
43	C1′	−211 (10)	2079 (7)	−2767 (11)	4.6 (0.3)
44	C2′	−59 (10)	1329 (7)	−2829 (11)	4.6 (0.3)
45	C3′	597 (10)	963 (7)	−3586 (11)	4.3 (0.3)
46	C4′	1108 (10)	1343 (7)	−4326 (12)	4.9 (0.3)
47	C5′	971 (11)	2090 (7)	−4272 (11)	5.0 (0.3)
48	C6′	326 (10)	2459 (7)	−3455 (11)	4.5 (0.3)
49	C7′	251 (10)	3270 (7)	−3376 (11)	4.7 (0.3)
50	C8′	1054 (11)	3655 (7)	−2706 (12)	5.5 (0.3)
51	C9′	1016 (10)	4408 (8)	−2608 (12)	5.3 (0.3)
52	C10′	186 (12)	4783 (8)	−3169 (12)	6.0 (0.3)
53	C11′	−598 (11)	4416 (7)	−3809 (12)	5.7 (0.3)
54	C12′	−543 (11)	3648 (8)	−3905 (11)	5.5 (0.3)
55	C13′	−972 (10)	2481 (8)	−1933 (11)	5.0 (0.3)

TABLE V. (continued)

No.	Atom	x	y	z	B_{eq} (Å^2)
56	C14′	−460 (11)	2408 (8)	−735 (12)	6.1 (0.3)
57	N15′	−1114 (10)	2798 (7)	210 (10)	6.5 (0.3)
58	C16′	−2200 (15)	2481 (12)	131 (17)	11.1 (0.5)
59	C17′	−551 (15)	2659 (12)	1319 (14)	10.0 (0.5)
60	C18′	−1368 (16)	3589 (9)	117 (16)	9.6 (0.4)
61	C19′	−396 (17)	4005 (10)	35 (17)	11.0 (0.5)
62	C20′	309 (12)	−176 (8)	−2921 (15)	7.2 (0.4)
63	C21′	790 (14)	−953 (8)	−3128 (16)	8.4 (0.4)
64	C22′	2156 (13)	1292 (9)	−5902 (14)	7.8 (0.4)
65	C23′	2515 (11)	4455 (9)	−1288 (15)	7.7 (0.4)
66	C24′	−690 (12)	5927 (8)	−3505 (15)	7.3 (0.4)
67	C25′	−1459 (13)	3232 (9)	−4539 (13)	7.2 (0.3)
68	C26′	−1720 (24)	3412 (16)	−5554 (19)	19.1 (0.8)
69	C27′	−2604 (21)	3149 (18)	−6435 (34)	23.7 (1.1)
70	O1′	814 (7)	222 (5)	−3684 (8)	6.0 (0.2)
71	O2′	1692 (7)	927 (5)	−5084 (8)	5.9 (0.2)
72	O3′	1759 (8)	4831 (5)	−1955 (9)	7.6 (0.2)
73	O4′	215 (8)	5539 (5)	−3030 (9)	7.0 (0.2)
74	O5′[a]	−2428 (20)	2621 (15)	−6675 (27)	12.4 (0.7)
75	O5″[a]	−3305 (21)	3325 (14)	−5937 (23)	10.9 (0.6)
76	W1[a]	891 (29)	1099 (20)	784 (33)	17.3 (1.0)
77	W2[a]	204 (33)	773 (23)	139 (32)	19.5 (1.2)

a) Atoms with half occupancy factor.

1.75 h. Work-up of the reaction mixture as noted above afforded pale yellow crystals (**6b**) (2.4 g, 75%), mp 89—90 °C (MeOH–hexane). IR cm^{-1}: 3550 (OH). ^{1}H-NMR δ: 2.44 (3H, s, NCH_3), 3.80 (3H, s, OCH_3), 5.84 (2H, s, OCH_2O), 6.46, 6.56 (each 1H, s, 2×ArH), 6.56—6.68 (3H, m, 3×ArH). *Anal.* Calcd for $C_{20}H_{23}NO_4$: C, 70.36; H, 6.79; N, 4.10. Found: C, 70.23; H, 6.79; N, 4.09.

General Procedure for Preparation of *o*-QAs (5)——$Pb(OAc)_4$ (1.1 eq) was added in one portion to an ice-cooled, stirred solution of **6** (100 mg) in CH_2Cl_2 (16 ml) and stirring was continued at the same temperature for 4—5 min. A precipitate was filtered off and H_2O (4—5 drops) was added to the filtrate. The mixture was swirled well and dried over anhydrous K_2CO_3 for 5 min. After filtration to remove the K_2CO_3, the solvent was removed at below 5 °C under vacuum to give *o*-QA (**5**) as an oil, quantitatively. As *o*-QA rapidly decomposed on standing at room temperature to give complex mixtures containing 4-acetoxytetrahydroisoquinolin-6-ols (**11**), work-up of the reaction mixture was carried out under ice-cooling and the *o*-QA was used without purification. The spectral data for **5** are listed in Table I.

General Procedure for Reaction of *o*-QA (5) with Ac_2O in the Presence of an Acid——(i) Without Solvent: *o*-QA (**5**), obtained from **6** (100 mg) as noted above, was dissolved in Ac_2O. Acid was added to the ice-cooled, stirred solution and stirring was continued at room temperature for an appropriate time. The reaction mixture was poured into ice-water. It was made alkaline with 5% aqueous $NaHCO_3$ and the product was taken up in $CHCl_3$. Usual work-up of the $CHCl_3$ extract furnished an oil, which was purified by preparative TLC. Spectral data, reaction conditions, yields and elemental analyses of **4** and **7** are listed in Tables II and III.

(ii) With Solvent: Ac_2O was added to an ice-cooled, stirred solution of *o*-QA (**5**), obtained from **6** (100 mg) as noted above, in an appropriate solvent and the acid was added to the mixture. The whole was stirred at room temperature. Work-up as noted above afforded **4** and/or **7**. The results are listed in Tables III and IV.

Preparation of Methoperchlorate (12)——A solution of **7a** (196 mg) and $NaBH_4$ (19 mg) in MeOH (3.5 ml) was stirred at room temperature for 50 min. K_2CO_3 (60.5 mg) was added to the mixture and the whole was refluxed for 45 min. After filtration to remove the K_2CO_3, H_2O was added to the filtrate and the product was taken up in $CHCl_3$. Usual work-up of the $CHCl_3$ extract afforded quantitatively an oil [IR cm^{-1}: 3525 (OH), 1620 ($NCOCH_3$). MS *m/z*: 417 (M^+)]. A mixture of the oil (84 mg), K_2CO_3 (40 mg) and EtI (0.1 ml) in EtOH (3 ml) was refluxed with stirring for 1.5 h. After filtration to remove the K_2CO_3, the solid was washed with hot benzene. Removal of the solvent from the combined organic layers *in vacuo* gave an oil (77 mg, 85.9%) [IR cm^{-1}: 3600, 3380 (OH), 1620 ($NCOCH_3$). MS *m/z*: 445 (M^+)]. A mixture of the crude product (70.8 mg) and $LiAlH_4$ (19 mg) in THF (5 ml) was stirred for 5 min and refluxed for 0.5 h. Usual work-up of the reaction mixture gave an oil (64 mg), which was purified by preparative TLC (developing solvent; $CHCl_3$: MeOH = 7 : 1) to give an oil (39 mg, 56.3%). IR cm^{-1}: 3300 (OH). ^{1}H-NMR δ: 0.95 (3H,

t, J=7.5 Hz, NCH_2CH_3), 1.49 (3H, t, J=7.5 Hz, OCH_2CH_3), 2.41 (3H, s, NCH_3), 3.81, 3.83, 3.90 (each 3H, s, 3×OCH_3), 6.61, 6.77 (each 2H, s, 4×ArH). MS m/z: 431 (M^+). A mixture of the oil (39 mg) and MeI (1 ml) in MeOH (3 ml) was refluxed for 3.5 h. Removal of the solvent gave a methiodide, which was treated with aqueous $NaClO_4$ to afford colorless needles (**12**) (27.8 mg, 56.3%), mp 153.5—154 °C (EtOH–ether). *Anal.* Calcd for $C_{26}H_{40}NO_5 \cdot ClO_4 \cdot 0.5H_2O$: C, 56.25; H, 7.26; N, 2.53. Found: C, 55.85; H, 7.13; N, 2.46.

Structural Determination of 12——(i) Crystal Data: $C_{26}H_{40}NO_5 \cdot ClO_4 \cdot 0.5H_2O$ (M_r=555.1); triclinic, space group, $P\bar{1}$, Z=4, unit cell dimensions; a=13.071 (7), b=18.633 (9), c=11.984 (6) Å, α=97.75 (5), β=94.39 (5), γ=87.06 (4)°, U=2881.1 Å^3, D_{cal}=1.280 g cm^{-3}.

(ii) X-Ray Crystallographic Analysis: A single crystal with approximate dimensions of 0.02×0.11×0.4 mm was chosen for the X-ray study from crystals grown in EtOH–ether solutions. The intensity data were obtained using Cu K_α radiation monochromated by the use of a graphite plate. The crystal data are given above (i). The intensities of 3150 reflections were measured as above the $2\sigma(I)$ level out of 5468 within the 2θ angle range of 6° through 120°. A set of trial structures was obtained by the direct method, and the one which agreed best with the Patterson function was refined by difference Fourier synthesis and least-squares calculations. The final refinement, including two solvate water molecules of half weight and the disordered hydroxyl group 05′ and 05″ gave an R value of 0.113.[12] Hydrogen atoms were not included. Atomic coordinates and equivalent isotropic temperature factors are given in Table V and an ORTEP drawing of **12** is depicted in Fig. 1.

The crystal contains two independent molecules in the asymmetric unit, and the two molecules are very similar to each other in dimensions and conformation.

2-Hydroxy-1,10,11-trimethoxy-6-methyl- and 2-Hydroxy-1-methoxy-6-methyl-10,11-methylenedioxyhomoaporphines (13a and 13b)——**13a**: A solution of **4a** (145 mg) and 10% HCl (10 ml) in MeOH (10 ml) was refluxed for 0.5 h. Removal of the solvent *in vacuo* gave an oil, which was basified with 5% aqueous $NaHCO_3$. The product was taken up in $CHCl_3$. Usual work-up of the $CHCl_3$ extract gave an oil, which was crystallized by trituration in a mixture of benzene and hexane to give pale yellow crystals (**13a**) (125.2 mg, 96.6%), mp 170—171 °C (benzene–hexane). IR cm^{-1}: 3550 (OH). ^{1}H-NMR δ: 2.34 (3H, s, NCH_3), 3.28 (3H, s, 1-OCH_3), 3.80, 3.86 (each 3H, s, 2×OCH_3), 6.58, 6.66, 6.96 (each 1H, s, 3×ArH). *Anal.* Calcd for $C_{21}H_{25}NO_4 \cdot 0.25C_6H_6$: C, 72.00; H, 7.07; N, 3.73. Found: C, 72.32; H, 7.11; N, 3.53. **13b**: A solution of **4b** (122 mg) and 10% HCl (10 ml) in MeOH (10 ml) was refluxed for 0.5 h. Work-up of the reaction mixture as noted for **13a** afforded pale yellow crystals (**13b**) (86 mg, 85%), mp 178—181 °C (hexane). IR cm^{-1}: 3520 (OH). ^{1}H-NMR δ: 2.40 (3H, s, NCH_3), 3.32 (3H, s, 1-OCH_3), 5.94, 5.96 (each 1H, d, J=0.8 Hz, OCH_2O), 6.67, 6.72, 6.94 (each 1H, s, 3×ArH). *Anal.* Calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13. Found: C, 70.68; H, 6.09; N, 4.10.

1,2,10,11-Tetramethoxyhomoaporphine (14a)——A solution of **13a** (100 mg) in MeOH (3 ml) was treated with excess CH_2N_2–ether solution (10 ml) at room temperature for 12 h. Removal of the solvent afforded an oil, which was purified by preparative TLC (developing solvent; $CHCl_3$: MeOH = 8 : 1) to furnish an oil (**14a**) (60.4 mg, 58%) and **13a** (22 mg). **14a**: ^{1}H-NMR δ: 2.40 (3H, s, NCH_3), 3.42 (3H, s, 1-OCH_3), 3.86, 3.88, 3.93 (each 3H, s, 3×OCH_3), 6.64, 6.73, 7.06 (each 1H, s, 3×ArH). MS m/z: 369 (M^+). **14a**·HCl, mp 243—245 °C (dec.) (MeOH–ether) (lit.[6] 242—244 °C (dec.)). The ^{1}H-NMR spectral data and the melting point of **14a**·HCl were identical with those given in the literature.[6]

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they proposed an analogous mechanistic pathway for the formation of (i).

11) In the casé of *o*-QAs (**1**), the formation of an intermediate such as **B**, which would lead to a noraldehyde-amide similar to **7**, might be unfavorable, because steric hindrance would be greater in the formation of the five-membered ring than in that of the six-membered one. Therefore, the noraldehyde-amide would not be formed in the similar reaction of **1**.

12) A list of *F*o and *F*c values may be obtained from one (Y.I.) of the authors upon request.

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Isoxazole Derivatives as Centrally Acting Muscle Relaxants. III.[1] Synthesis and Activity of Conformationally Restricted Analogs[2]

TOCHIRO TATEE,*,[a] KAZUHISA NARITA,[a] SHUJI KURASHIGE,[a] SHINJI ITO,[a] HIROSHI MIYAZAKI,[a] HIROSHI YAMANAKA,[b] MICHINAO MIZUGAKI,[b] TAKAO SAKAMOTO,[b] and HIDEOMI FUKUDA[c]

Research Laboratories of Pharmaceuticals Group, Nippon Kayaku Co., Ltd.,[a] Shimo, Kita-ku, Tokyo 115, Japan, Pharmaceutical Institute, Tohoku University,[b] Aobayama, Sendai 980, Japan, and Faculty of Pharmaceutical Sciences, The University of Tokyo,[c] Hongo, Bunkyo-ku, Tokyo 113, Japan

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Conformationally restricted analogs of muscle-relaxant 3-amino-2-methyl-*N*-(3-phenyl-5-isoxazolyl)propanamides (**1**) and 5-(3-aminopropylamino)-3-phenylisoxazoles (**2**) were prepared, and their muscle-relaxant and other pharmacological activities were tested and compared with those of the corresponding acyclic derivatives. 7-(3-Diethylamino-2-methylpropanoyl)-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (**8**) exhibited muscle-relaxant and anticonvulsant activities comparable with those of corresponding acyclic derivatives, *i.e.* 3-diethylamino-2-methyl-*N*-(3-phenyl-5-isoxazolyl)propanamides (**1e—g**), but other types of compounds showed decreased activities. The preferred conformation of the present isoxazole derivatives for muscle-relaxant activity is discussed.

7-Benzyl-6-methyl-3-phenyl-4-pyrrolidino-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (**3d**) showed moderate central nervous system-depressant activity.

Keywords——muscle relaxant; isoxazole derivative; isoxazolo[5,4-*b*]pyridine; structure–activity relationship; conformation; conformationally restricted analog; tolperisone; anticonvulsant; anemic decerebrate rigidity

In the previous papers,[1] we reported the preparation, pharmacological activity, and quantitative structure–activity relationships (QSAR) of 5-aminoisoxazole derivatives (**1**, **2**) containing a 3-aminopropanamide or 3-aminopropylamino side chain as centrally acting muscle relaxants. Both types of isoxazole derivatives (**1**, **2**) have significant muscle-relaxant activities. However, the derivatives with a 3-aminopropylamino side chain showed weaker anticonvulsant activity and higher depressant action on spontaneous motor activity than the derivatives with a 3-aminopropanamide side chain. Among the derivatives with a 3-aminopropanamide side chain synthesized, 3-diethylamino-2,*N*-dimethyl-*N*-(3-phenyl-5-isoxazolyl)propanamide (**1a**) was obtained as an optimized derivative with selective action, namely potent muscle-relaxant and anticonvulsant activities with reduced central nervous system (CNS)-depressant activity.

1 **2**

Chart 1

	R^1	NR^2R^3
3a	H	N◯O
3b	H	N◯
3c	H	N◯
3d	CH_2Ph	N◯

4a : R = H
4b : R = *p*-Ts
4c : R = COPh
4d : R = CH_2Ph

5a : R = H
5b : R = Me

6a : R = Me
6b : R = Et
6c : R = Pr
6d : R = iso-Pr

7

8

Chart 2

In these 3-amino-*N*-(5-isoxazolyl)-2-methylpropanamides (**1**) and 5-[(3-aminopropyl)-amino]isoxazoles (**2**), the 3-aminopropanamide and 3-aminopropylamino side chains can rotate freely. Recently, conformationally restricted analogs of pharmacologically active compounds were prepared and their activities were examined in order to identify the preferred conformation at the site of action.[3] The compounds with one of the carbon atoms on the side chain connected with the isoxazolyl ring carbon, or another carbon atom on the side chain, or the amide nitrogen atom should have comparable activities with their corresponding acyclic derivatives, if they have the spatial interrelationship of functional groups that is necessary for activity. A side effect can be eliminated if it is caused by a conformation different from that required for the principal effect.[3e] From this point of view, we synthesized conformationally restricted analogs (**3**—**8**) of the previously reported isoxazole derivatives, and compared their muscle-relaxant and anticonvulsant activities with those of the corresponding acyclic analogs. We found compound **8** to possess a muscle-relaxant activity comparable with those of the corresponding acyclic derivatives. Compound **3d** exhibited moderate CNS-depressant activity instead of the muscle-relaxant activity. In the present paper, we describe the synthesis of **3**—**8** and their pharmacological activities. The preferred conformation of muscle-relaxant isoxazole derivatives at the site of action is discussed.

Synthesis

4-Substituted isoxazolo[5,4-*b*]pyridines (**3a**—**c**) were synthesized by reductive cyclization of the 3-(5-isoxazolylamino)butanamides (**9a**—**c**) with sodium bis-(2-methoxyethoxy)-aluminum hydride (SMEAH) in toluene. Compound **3c** was reacted with benzyl chloride

Chart 3

Chart 4

in the presence of sodium amide in toluene to give a benzylated derivative (**3d**). The 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **3d** is best interpreted on the basis of a *trans* stereostructure of the 4-pyrrolidino group and 6-methyl group; J (4-H, 5-axial H) = 3.4 Hz, J (4-H, 5-equatorial H) = 3.4 Hz, J (5-axial H, 5-equatorial H) = 14.4 Hz, J (5-axial H, 6-H) = 11.2 Hz, J (5-equatorial H, 6-H) = 2.9 Hz.

5-Substituted isoxazolo[5,4-*b*]pyridines (**4**, **5**) were synthesized *via* 5-ethoxycarbonyl derivatives (**12a**, **b**). Namely, benzoylacetonitrile was heated with diethyl alkylidenemalonates in ethanol to give Michael adducts (**10a**, **b**), which were converted into isoxazole diesters (**11a**, **b**) by reaction with hydroxylamine hydrochloride and pyridine in ethanol. Compounds **12a**, **b** were synthesized by refluxing **11a**, **b** with sodium methoxide in methanol.

When the amido-ester (**12a**) was treated with borane in tetrahydrofuran (THF), the amino alcohol (**13a**) was obtained. The amino alcohol (**13a**) reacted readily with *p*-toluenesulfonyl chloride (*p*-TsCl) in pyridine to afford the ditosylate (**13b**) and the monoto-

12c : R = H
12d : R = Me
12e : R = H
12f : R = Me
5a : R = H
5b : R = Me

Chart 5

6a : R = Me
6b : R = Et
6c : R = Pr
6d : R = iso-Pr

Chart 6

sylate (**13c**), which were converted into **4b** and **4a**, respectively, by reaction with pyrrolidine.

When the amido-ester (**12a**) was treated with lithium borohydride in 1,2-dimethoxyethane, the diol (**11c**) was obtained as a major product along with the amido-alcohol (**12c**) and the amino alcohol (**13a**). When the 4-methylated amido-ester (**12b**) was reacted with the same reagent, the amido-alcohol (**12d**) was obtained as a sole product. In a 4-methylated isoxazolo[5,4-*b*]pyridine ring system such as that of **12b**, the methyl group at position 4 may be oriented quasi-axial due to repulsion of the phenyl group at position 3 and thus protect the amide function from hydride attack. Therefore, the amidoalcohols (**12c**, **d**) were tosylated with *p*-TsCl in pyridine, followed by reaction with dimethylamine to give **5a**, **b**, respectively.

N-(5-Isoxazolyl)nipecotamides (**6a**—**d**) were synthesized by the reaction of the corresponding *N*-substituted nipecotates[4] with the magnesium salt of 5-methylamino-3-phenylisoxazole (**14b**).

Among unsymmetrically N,N'-disubstituted hexahydro-1*H*-1,4-diazepin-5-ones, the preparation of 4-cyclohexyl-1-(1,2-diphenylethyl)hexahydro-1*H*-1,4-diazepin-5-one has been reported.[5] A similar scheme was devised to prepare a 4-(5-isoxazolyl)hexahydro-1*H*-1,4-diazepin-5-one (**7**). The chloroacetamide derivative (**14c**) was reacted with ethylamine to give the 2-ethylaminoacetamide derivative (**14d**), which was then converted into 5-(2-ethylaminoethyl)amino-3-phenylisoxazole (**14e**) by reduction with lithium aluminum hydride in THF. Compound **14e** was reacted with an equimolar amount of *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl)thiocarbonate and triethylamine in chloroform to give a *tert*-butoxycarbonyl (Boc) derivative (**14f**), which was acylated to yield a 2-propenamide (**14g**) by the use of methacryloyl chloride and triethylamine. The Boc group of **14g** was eliminated by treatment with trifluoroacetic acid to give the 2-propenamide derivative (**14h**), which was cyclized to **7** by heating with acetic acid in benzene.

3-Phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (**13d**) was synthesized by hydrolysis and concomitant decarboxylation of the amido-ester (**12a**) with potassium carbonate in ethanol, followed by the reduction of the resulting amide (**12g**) with lithium aluminum

Chart 7

Chart 8

conformationally restricted analogs — corresponding acyclic analogs — conformationally restricted analogs — corresponding acyclic analogs

type I: 3a—d ----- 2a—c

type II: 4a—d ----- 2d, e

type III: 5a ----- 1b

type IV: 6a—d ----- 1a,c,d

type V: 7 ----- 1a—d

type VI: 8 ----- 1e—g

Chart 9

hydride. Compound **13d** reacted readily with methacryloyl chloride under basic conditions to give the 2-propenamide (**13e**), which was converted to the 3-diethylamino-2-methylpropanamide (**8**) by Michael addition with diethylamine.

The yields, melting points, selected spectroscopic data, and the results of elemental analysis of compounds **3a—d**, **15a—f**, and of the free bases or fumarates of compounds **1e, f** are listed in Tables V and VI in the experimental section.

Pharmacology

Six types of conformationally restricted analogs were thus prepared and their activities were compared with those of the corresponding acyclic analogs.

Pharmacological and toxicological data for compounds **3a—d**, as well as the corresponding acyclic analogs (**2a—c**) are shown in Table I. Muscle-relaxant and anticonvulsant activities of **2a—c** and **3a—d** were tested by means of the traction test, and the pentylenetetrazole (PTZ) test, respectively. The results for **3a—c** indicated that derivatives with a hydrogen as R did not show any muscle-relaxant activity. Compound **3d** (with a benzyl group as R) exhibited higher potency than **3c**. Muscle-relaxant and anticonvulsant activities of these bicyclic isoxazole derivatives were compared with those of the corresponding acyclic compounds **2a—c**. Compounds **3c** and **3d** are rigid analogs of **2a** and **2b**, respectively; namely, the carbon atom at the 3-position of the 3-aminopropylamino side chain of compounds **2a** and **2b** is connected with the isoxazolyl 4-position in compounds **3c** and **3d**. Although **3c** and **3d** showed moderate anticonvulsant activities, both derivatives showed decreased potency in muscle relaxation.

CNS-depressant activity was evaluated by the effect on the conditioned-avoidance

response and the depression of spontaneous motor activity determined by the revolving wheel method. The rigid compounds (**3c**, **d**) showed an increased CNS-depressant activity. Compound **3c** showed weak potency in the depression in motor activity and the benzylated derivative (**3d**) exhibited increased potency in the conditioned avoidance response and motor activity. Acyclic compounds (**2a**, **b**) showed less CNS-depressant activity.

The isoxazolopyridine derivatives **4a** and **4d** are also rigid analogs of **2d** and **2e**, respectively; namely, the carbon atom of the methyl group on the 3-aminopropylamino side chain of compounds **2d** and **2e** is connected with the isoxazolyl 4-position in compounds **4a**

Table I. Pharmacological Data on Isoxazolopyridines (**3a**—**d**) and the Corresponding Acyclic Analogs (**2a**—**c**)

3a—**d** **2a**—**c**

Compd. No.	T[a] i.p.	PTZ[b] s.c.	C[c] i.p.	D[d] s.c.	LD_{50}[e] i.p.
3a	>300	f)	>100	f)	>1000
3b	>300	f)	>300	f)	>1000
3c	100	50—100	>30	10—30	84.5
3d	50	25—50	10—30	3	126
2a	10—30	>50	f)	f)	100—300
2b	10	>100	100	30	100—300
2c	30	>100	>100	f)	100—300
Tolperisone	100	13.7	100	30	180
Chlorpromazine	f)	f)	3—10	0.5—1.0	108

a—*d*) Activity is presented as ED_{50}. *a*) Traction test in mice. *b*) Anticonvulsant activity was examined against tonic extensor convulsion induced by PTZ. *c*) Conditioned avoidance response. *d*) Depression in motor activity determined by the revolving wheel method. *e*) 50% lethal dose in mice. *f*) Not tested.

Table II. Pharmacological Data on Isoxazolopyridines (**4a**—**d**) and the Corresponding Acyclic Analogs (**2d**, **e**)

4a—**d** **2d**, **e**

Compd. No.	T[a] i.p.	MES[b] i.p.	C[a] i.p.	D[a] i.p.	LD_{50}[a] i.p.
4a	>50	>50	>30	>30	72.6
4b	>300	c)	>300	300	1000
4c	>300	100—300	>100	100	c)
4d	>50	>50	>10	>30	49.5
2d	>30	c)	c)	>30	100—300
2e	30	c)	c)	30	300
Tolperisone	50—100	50—100	100	63	100—300
Chlorpromazine	c)	c)	3—10	1—2	108

a) See footnote to Table I. *b*) Anticonvulsant activity was examined against maximal electroshock (MES). *c*) Not tested.

and **4d**. Anticonvulsant activity was tested by means of the maximal electroshock (MES) test. These compounds (**4a** and **4d**) did not show any significant pharmacological activity. Even the benzylated compound (**4d**) was less active than the corresponding acyclic compound (**2e**) in terms of the traction test.

The 6-oxoisoxazolopyridine derivative (**5a**) corresponds to the propanamide (**1b**); namely, the carbon atom of the methyl group on the 3-aminopropanamide side chain of compound **1b** is connected with the isoxazolyl 4-position in compound **5a**. The pharmacological data are shown in Table III. Compound **5a** showed less than half of the muscle-relaxant and anticonvulsant activities of **1b**. The 4-methyl homolog (**5b**) exhibited decreased effects.

In compounds **6a**—**d**, the carbon atom of the methyl group on the 3-aminopropanamide

Table III. Pharmacological Data on Isoxazolopyridines (**5a, b**) and the Corresponding Acyclic Analogs (**1b**)

5a, b **1b**

Compd. No.	T[a] i.p.	PTZ[a] i.p.	MES[b] i.p.	C[a] i.p.	LD_{50}[a] i.p.
5a	>200	>200	>200	[c]	[c]
5b	>400	>400	>400	>100	600
1b	100	50	50—100	[c]	300—1000

a) See footnote to Table I. *b*) See footnote to Table II. *c*) Not tested.

Table IV. Pharmacological Data on Conformationally Restricted Analogs (**6a**—**d**, **7**, **8**) and the Corresponding Acyclic Analogs (**1a, c**—**g**)

6a—**d** **7** **8** **1a, c**—**g**

Compd. No.	R[a] Inhibition ratio (%) at 2.5 mg/kg i.v.	R[a] Inhibition ratio (%) at 5 mg/kg i.v.	PTZ ED_{50} (mg/kg) i.p.
6a	17	[b]	[b]
6b	16	[b]	50
6c	5	[b]	[b]
6d	8	[b]	[b]
7	[b]	6	56.1
8	28	52	12.5
1a	46	63	14.0
1c	20	59	13.2
1d	26	76	28.1
1e	28	76	12.5
1f	29	65	50
1g	28	62	25—50

a) Muscle relaxant activity was tested in the anemic decerebrate rigidity model. *b*) Not tested.

side chain in compounds **1a, c, d** is connected with the carbon atom of the alkyl substituent on the 3-amino group. In compound **7**, the carbon atom of the alkyl substituent on the 3-amino group of the 3-aminopropanamide side chain of compounds **1a—d** is connected with the amide nitrogen, or the carbon atom of the alkyl substituent on the amide nitrogen. In compound **8**, the carbon atom of the alkyl substituent on the amide nitrogen of compounds **1e—g** is connected with the isoxazolyl 4-position. Pharmacological data on **6a—d**, **7**, and **8** are shown in Table IV. Muscle-relaxant activities were tested in the anemic decerebrate rigidity model,[1a)] and are shown as an inhibition ratio at 2.5 mg/kg or 5 mg/kg i.v.

Compounds **6b** and **6c** showed decreased potency at 2.5 mg/kg i.v. in the rigidity model, compared with their acyclic derivatives (**1a** and **1c**), respectively. The isoxazolyl diazepinone (**7**) inhibited the tension of rigid forelimbs by only 6% at 5 mg/kg in the rigidity model,[1a)] and showed weak anticonvulsant activity. The isoxazolopyridine derivative (**8**) showed muscle-relaxant and anticonvulsant activities comparable with those of the corresponding acyclic compound **1e** or **1g**.

Discussion

Derivatives of types I—V showed decreased potency compared with the corresponding acyclic analogs, and only compound **8** showed comparable activity. As mentioned in the previous paper, a good correlation equation was obtained between potency and hydrophobic parameters, but the equation can only explain less than 70% of the variance of activity.[1b)] We were therefore interested in the activity-determining factors other than the hydrophobic parameter. The mechanisms of action of centrally acting muscle relaxants are still uncertain, and the nature of the preferred conformation of the present isoxazole derivatives at the site of action is not known. However, the spatial interrelationships of the terminal amino group, the 5-isoxazolyl amino or amide group, the isoxazole ring, and the phenyl group are expected to play an important role in the activity.[3)]

Among compounds of types I—VI, type I had the least freedom of interrelationship of these four groups, and in types II and III, the terminal amino group can rotate only in a limited area. In types IV and V, alkyl substituents on the terminal amino group are conformationally fixed. The decreased potency of the rigid analogs of these types indicates that other conformations than types I—V are desirable for activity.

In type VI, the terminal amino group can move freely except in the area that is occupied by the 4,5,6-positions of the isoxazolo[5,4-*b*]pyridine ring system, where the terminal amino groups of the compounds of types I—III are located, and only the alkyl group on the amide nitrogen is fixed *cis* to the isoxazolyl 4-hydrogen.

The present results suggest that the preferred conformation of the isoxazole derivatives as muscle relaxants may be close to type VI, and the terminal amino group may not be located near the isoxazolyl 4-position.

Experimental

Melting points were measured on a Sibata apparatus and are not corrected. Infrared (IR) spectra were determined on a JASCO model IR-G or a JASCO model A-202 spectrometer, taken as neat film, Nujol mull, or KBr disc as indicated in parentheses. Data are presented in reciprocal centimeters and only the important diagnostic bands are reported. ^{1}H-NMR spectra were obtained with a JEOL JNM-PMX60 (60 MHz) spectrometer, unless otherwise noted. The 400 MHz ^{1}H-NMR spectra were measured on a JEOL JNM-GX 400 spectrometer. Chemical shifts are expressed downfield from tetramethylsilane (TMS) as an internal standard. Data are presented in the form: value of signal (integrated number of protons, peak multiplicity, coupling constant (if any)). Mass spectra (MS) were taken on a Shimadzu 7000 mass spectrometer operating at 70 eV unless otherwise indicated. High-resolution MS were taken on a VG Analytical MM ZAB 2F-HF.

Unless otherwise stated, the reaction mixture was quenched with H_2O, and thoroughly extracted with organic

solvent. The extract was washed with saturated brine, dried, and filtered. The solvent was removed by rotary evaporation. The extraction solvent and drying agent are indicated in parenthesis. For column chromatography, Merck Kieselgel 60 (No. 7734) was used. For alumina dry column chromatography, Sumitomo Active Alumina KCG-30 was uniformly mixed with Merck Fluorescent-indicator F_{254} (No. 9182). For silica gel dry column chromatography, Woelm Pharma No. 4526 was used. For preparative thin layer chromatography, Merck Kieselgel 60 F_{254} No. 5717 was used. The chromatographic solvent is presented in parenthesis.

Synthesis

6-Methyl-3-phenyl-4-pyrrolidino-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (3c)——An excess of 70% solution of SMEAH in PhMe (500 ml) was added dropwise at room temperature to a suspension of *N*-[3-(3-phenyl-5-isoxazolyl)aminobutanoyl]pyrrolidine (**9c**, 230 g, 1.3 mol) in PhMe (1.5 l). The reaction mixture was stirred at room temperature for 12 h, then quenched with H_2O. Usual product isolation (PhMe, $MgSO_4$) gave an oil, which crystallized, and was recrystallized to afford **3c** (78.3 g) as a colorless powder. MS *m/z*: 283 (M^+). Compounds **3a, b** were obtained as colorless powders in the same manner from **9a, b**, respectively.

7-Benzyl-6-methyl-3-phenyl-4-pyrrolidino-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (3d)——Compound **3c** (28.34 g, 100 mmol) was added in portions to a suspension of $NaNH_2$ (5.85 g) in anhydrous PhMe (300 ml). The reaction mixture was heated under reflux for 1 h, then cooled to room temperature, and benzyl chloride (15.43 g, 122 mmol) was added dropwise. Stirring was continued at room temperature for 12 h, and product isolation (PhMe, $MgSO_4$) gave an oil which was purified on a column of silica gel ($CHCl_3$–MeOH, 10 : 1) to afford **3d** (10.95 g) as colorless needles, mp 95—98 °C (hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, d, J=7 Hz), 1.35 (10H, m), 3.4—3.9 (2H, m), 4.59 (2H, AB quartet, J=15.4 Hz), 7.1—7.9 (10H, m). MS *m/z*: 373 (M^+), 202, 189, 186, 92. *Anal.* Calcd for $C_{24}H_{27}N_3O$: C, 77.18; H, 7.29; N, 11.25. Found: C, 76.94; H, 7.33; N, 11.06. Compound **3d** was converted into the hydrochloride in a usual manner to give **3d**·HCl as a colorless powder. IR (KBr) ν: 2580, 2500, 1615 cm^{-1}

5-Ethoxycarbonyl-4-methyl-6-oxo-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (12b)——A mixture of benzoylacetonitrile (3 g, 20.7 mmol) and diethyl ethylidenemalonate (4 g, 21.5 mmol) in EtOH (70 ml) was heated under reflux for 8 h. The reaction mixture was evaporated to give an oil (7.1 g), which was purified by dry column chromatography on silica gel (400 g, hexane–Et_2O, 2 : 1 followed by 1 : 1) to afford diethyl 3-cyano-2-methyl-4-oxo-4-phenylbutan-1,1-dicarboxylate (**10b**, 5.01 g, 73%) as an oil. IR (neat) ν: 2250, 2200, 1755, 1745, 1740, 1730, 1695 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, t, J=7 Hz), 1.33 (3H, t, J=7 Hz), 1.33 (3H, d, J=7 Hz), 2.8—3.9 (2H, m), 4.27 (2H, q, J=7 Hz), 4.33 (2H, q, J=7 Hz), 5.15 (1H, d, J=4 Hz), 7.3—8.2 (5H, m). MS *m/z* (20 eV, relative intensity): 331 (M^+, 1.3), 141 (22), 115 (19), 106 (28), 105 (100).

A mixture of **10b** (500 mg, 1.51 mmol), $NH_2OH \cdot HCl$ (114 mg, 1.64 mmol), and pyridine (300 mg, 3.79 mmol) was heated under reflux for 3 h. $NH_2OH \cdot HCl$ (230 mg, 3.31 mmol) and pyridine (200 mg, 2.53 mmol) were added, and the refluxing was continued for another 1 h. The reaction mixture was quenched with H_2O and product isolation

TABLE V. 4-Amino-6-methyl-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridines (**3a**—**d**)

Compd. No.	R	NR_2	Recrystn. solvent	mp (°C)	Yield	Formula	Analysis (%) Calcd (Found)		
							C	H	N
3a	H	N O	Et_2O	152—154	20[a]	$C_{17}H_{21}N_3O_2$	67.88 (68.20	7.20 7.07	14.25 14.04)
3b	H	N	Et_2O	137—140	27[a]	$C_{18}H_{23}N_3O$	72.62 (72.69	7.66 7.80	14.04 14.13)
3c	H	N	MeOH	157—158	36[a]	$C_{17}H_{21}N_3O$	72.33 (72.05	7.15 7.47	15.08 14.83)
3d	$PhCH_2$	N	EtOH[b]	138—140[b]	29[c]	$C_{24}H_{27}N_3O \cdot HCl$[b]	70.12 (70.31	7.20 6.88	10.14[b] 10.25)

a) Yield of cyclization. *b*) As the hydrochloride. *c*) Yield of 7-benzylation.

(AcOEt, $MgSO_4$) gave an oil (507 mg), which was purified by silica gel preparative thin layer chromatography (PhMe–Et_3N, 3:1) to afford diethyl 2-methyl-2-(5-amino-3-phenyl-4-isoxazolyl)ethan-1,1-dicarboxylate (**11b**, 265 mg, 51%) as an oil. IR (Nujol) ν: 3380, 3150, 1745, 1680, 1653 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.10 (3H, d, J=7 Hz), 1.17 (3H, t, J=7 Hz), 1.23 (3H, t, J=7 Hz), 3.2—3.8 (2H, m), 4.10 (2H, q, J=7 Hz), 4.15 (2H, q, J=7 Hz), 4.8 (2H, br s), 7.45 (5H, s). MS m/z (relative intensity): 346 (M^+, 24), 188 (12), 187 (100), 159 (12), 69 (11).

A mixture of **11b** (230 mg, 0.665 mmol) and NaOMe (50 mg, 0.93 mmol) in MeOH (8 ml) was heated under reflux for 1.5 h. The reaction mixture was evaporated and the residue was diluted with H_2O and acidified with dilute HCl. Product isolation (AcOEt, $MgSO_4$) gave an oil (206 mg), which was purified by silica gel preparative thin layer chromatography (hexane–Et_2O, 1:2) to afford **12b** (185 mg, 93%) as colorless plates, mp 145—146 °C (hexane–AcOEt). IR (Nujol) ν: 1740, 1690, 1655 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.20 (3H, t, J=7 Hz), 1.25 (3H, d, J=7 Hz), 3.4—4.0 (2H, m), 4.17 (2H, q, J=7 Hz), 7.3—7.9 (5H, m), 9.4 (1H, br s). MS m/z (relative intensity): 300 (M^+, 10), 227 (100), 105 (24), 77 (30), 51 (23). *Anal.* Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 63.59; H, 5.15; N, 9.17.

5-Ethoxycarbonyl-6-oxo-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (12a)——Compound **12a** was obtained as colorless needles in the same manner in 24% yield from benzoylacetonitrile, mp 179—180 °C (hexane–AcOEt). IR (Nujol) ν: 1740, 1690, 1660 cm^{-1}. ^1H-NMR (pyridine-d_5) δ: 1.20 (3H, t, J=7 Hz), 3.1—4.3 (3H, m), 4.27 (2H, q, J=7 Hz), 7.4—8.2 (5H, m). MS m/z (relative intensity): 286 (M^+, 10), 215 (50), 214 (54), 213 (100), 105 (77), 104 (35). *Anal.* Calcd for $C_{15}H_{14}N_2O_4$: C, 62.93; H, 4.93; N, 9.79. Found: C, 63.05; H, 4.70; N, 9.75.

5-Hydroxymethyl-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (13a)——A solution of 1 M borane–THF complex (130 ml) was added dropwise to a solution of **12a** (25 g, 88 mmol) in anhydrous THF (600 ml) under N_2 and ice cooling. The mixture was stirred at room temperature for 3 h, then another 130 ml of the same borane complex was added under cooling, and stirring was continued for 17 h. The mixture was quenched with H_2O and product isolation (AcOEt, $MgSO_4$) afforded the residue (26.3 g), which was purified by silica gel dry column chromatography (500 g, Et_2O) to give unreacted **12a** (1.53 g, 6.1%), and **13a** (9.93 g, 52% yield from the reacted material) as colorless plates, mp 144—145 °C (EtOH). IR (Nujol) ν: 3300, 1635 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.6—2.2 (1H, m), 2.2—2.7 (2H, m), 2.7—3.7 (5H, m), 4.6 (1H, br s), 7.3—7.8 (5H, m). MS m/z (20 eV, relative intensity): 230 (M^+, 100), 127 (20), 99 (32).

3-Phenyl-5-pyrrolidinomethyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (4a) and 3-Phenyl-5-pyrrolidinomethyl-7-(*p*-toluenesulfonyl)-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (4b)——*p*-TsCl (12.0 g, 62.9 mmol) was added to a solution of **13a** (11.1 g, 48.2 mmol) in anhydrous pyridine (50 ml) under ice cooling. The mixture was stirred at room temperature for 13 h, then *p*-TsCl (3.0 g, 15.8 mmol) was added and the stirring was continued for another 24 h. The mixture was evaporated and the residue (41.2 g) was separated by dry column chromatography on silica gel (1 kg, CH_2Cl_2) to afford 3-phenyl-7-(*p*-toluenesulfonyl)-5-(*p*-toluenesulfonyloxymethyl)-4,5,6,7-tetrahydroisoxazolo-[5,4-*b*]pyridine (**13b**, 5.62 g, 21.7%) as an oil, and 3-phenyl-5-(*p*-toluenesulfonyloxymethyl)-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (**13c**, 10.48 g, 56.6%) as an oil. Then, compound **13b** (5.62 g, 10.4 mmol) was mixed with pyrrolidine (50 ml) and heated under reflux for 3 h. The mixture was evaporated and the product was isolated (CH_2Cl_2, $MgSO_4$) as a colorless powder, which was recrystallized from $CHCl_3$–EtOH to give **4b** (3.34 g, 15.9% from **13a**) as colorless needles, mp 177—179 °C (AcOEt). IR (Nujol) ν: 1620, 1160 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.6—2.0 (5H, m), 2.2—2.7 (7H, m), 2.40 (3H, s), 3.0—3.5 (2H, m), 4.25 (1H, m), 7.2—8.0 (5H, m). MS m/z (20 eV, relative intensity): 437 (M^+, 1), 282 (19), 103 (8), 85 (10), 84 (100). *Anal.* Calcd for $C_{24}H_{27}N_3O_3S$: C, 65.88; H, 6.22; N, 9.60; S, 7.33. Found: C, 65.65; H, 6.00; N, 9.31; S, 7.29. Compound **4b** was converted into the hydrochloride in a usual manner to give **4b**·HCl as colorless needles, mp 208—209 °C (EtOH). IR (Nujol) ν: 2450, 1635 cm^{-1}. *Anal.* Calcd for $C_{24}H_{28}ClN_3O_3S$: C, 60.81; H, 5.95; Cl, 7.48; N, 8.87; S, 6.76. Found: C, 60.45; H, 5.71; Cl, 7.73; N, 8.58; S, 6.70.

Compound **13c** (10.48 g, 27.3 mmol) was mixed with pyrrolidine (70 ml) and refluxed for 3 h. The mixture was evaporated, and a usual product isolation gave the residue (16.93 g), which was purified by dry column chromatography on Al_2O_3 (400 g, CH_2Cl_2) to afford **4a** (6.08 g, 44.6% from **13a**) as colorless plates, mp 124—127 °C (PhH). IR (Nujol) ν: 3150, 1635 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.6—2.0 (4H, m), 2.0—2.8 (9H, m), 2.8—3.8 (2H, m), 4.7 (1H, br s), 7.2—7.9 (5H, m). MS m/z (relative intensity): 283 (M^+, 3), 225 (10), 214 (11), 212 (15), 84 (100). *Anal.* Calcd for $C_{17}H_{21}N_3O$: C, 72.05; H, 7.47; N, 14.83. Found: C, 71.95; H, 7.66; N, 15.14. Compound **4a** was converted into the hydrochloride in a usual manner to give **4a**·HCl as a colorless powder, mp 175—178 °C (MeOH). IR (Nujol) ν: 2580, 2480, 1650 cm^{-1}.

7-Benzoyl-3-phenyl-5-pyrrolidinomethyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (4c)——A mixture of **4a** (10.05 g, 35.5 mmol), PhCOCl (7.50 g, 53.4 mmol), and pyridine (30 ml) was allowed to stand overnight at room temperature. The product was filtered off, and washed with $CHCl_3$, AcOEt, 5% aqueous $KHCO_3$, 1 N HCl, and H_2O to give **4c**·HCl (6.08 g, 40.4%) as colorless plates, mp 217—219 °C. IR (Nujol) ν: 2600, 2500, 1660, 1620 cm^{-1}. MS m/z (relative intensity): 387 (M^+ of **4c** as a free base, 4), 213 (5), 105 (8), 85 (7), 84 (100). *Anal.* Calcd for $C_{24}H_{26}ClN_3O_2$: C, 67.99; H, 6.18; N, 9.91. Found: C, 67.84; H, 6.04; N, 10.00. The combined aqueous layer was basified with dilute NaOH, the product was isolated ($CHCl_3$, $MgSO_4$) and the residue (9.04 g) was purified by dry column chromatography on Al_2O_3 (500 g, light petroleum–CH_2Cl_2, 2:1) to give **4c** (2.26 g, 16.4%) as an oil. IR (neat)

ν: 1710, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.5—2.1 (5H, m), 2.3—2.9 (5H, m), 3.2—3.8 (5H, m). MS *m/z* (relative intensity): 387 (M^+, 4), 225 (4), 213 (4), 105 (8), 85 (7), 84 (100).

7-Benzyl-3-phenyl-5-pyrrolidinomethyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (4d)——$LiAlH_4$ (1.50 g, 39.5 mmol) was added in portions to a solution of **4c**·HCl (2.8 g, 6.6 mmol) in anhydrous THF (150 ml) under N_2 and ice cooling. The mixture was stirred at room temperature for 5 h, the reaction mixture was quenched with H_2O, and the product was isolated (AcOEt, $MgSO_4$). HCl gas was passed through a solution of the residue (3.20 g) in AcOEt (25 ml) and the product was recrystallized to give **4d**·HCl (2.02 g, 74.6%), mp 170—172 °C (AcOEt–MeOH) as a colorless powder. IR (Nujol) ν: 2600, 1650, 1605 cm^{-1}. *Anal.* Calcd for $C_{24}H_{28}ClN_3O$: C, 70.31; H, 6.88; Cl, 8.65; N, 10.25. Found: C, 70.22; H, 6.96; Cl, 8.71; N, 10.20. Compound **4d**·HCl was dissolved in H_2O, basified with $NaHCO_3$, and extracted with AcOEt. The organic layer was dried over $MgSO_4$, evaporated, and recrystallized from hexane–AcOEt to give **4d** as colorless needles, mp 121—123 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.2—2.2 (5H, m), 2.2—3.0 (4H, m), 3.0—3.9 (6H, m), 4.65 (2H, s), 7.2—7.9 (10H, m). MS *m/z* (relative intensity): 225 (11), 214 (9), 212 (11), 180 (7), 152 (7), 84 (100). *Anal.* Calcd for $C_{24}H_{27}N_3O$: C, 77.18; H, 7.29; N, 11.25. Found: C, 77.31; H, 7.24; N, 10.89.

5-Hydroxymethyl-6-oxo-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (12c)——$NaBH_4$ (9.20 g, 243 mmol) and LiBr (20.00 g, 230 mmol) were added in portions to a solution of **12a** (30.1 g, 105 mmol) in 1,2-dimethoxyethane (550 ml) under ice cooling, and the stirring was continued at room temperature for 5 h. The reaction mixture was quenched with H_2O, and neutralized with dilute HCl. Product isolation (AcOEt, $MgSO_4$) gave an oil, which crystallized, and was recrystallized from CH_2Cl_2 to afford **12c** (4.26 g, 16.6%). The mother liquor was evaporated and the residue (25.68 g) was separated by column chromatography on silica gel (1 kg, hexane–AcOEt, 1 : 1, next AcOEt alone) to give **13a** (2.81 g, 11.6%) and **12c** (2.15 g, 8.4%) as colorless plates, mp 196—197 °C (EtOH). IR (Nujol) ν: 3400, 1690, 1655 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 3.22 (3H, br s), 4.3—4.5 (2H, m), 7.3—8.2 (5H, m). MS *m/z* (relative intensity): 244 (M^+, 7), 169 (25), 105 (100), 104 (28), 77 (25). *Anal.* Calcd for $C_{13}H_{12}N_2O_3$: C, 63.92; H, 4.95; N, 11.47. Found: C, 63.91; H, 4.91; N, 11.39. Further elution with AcOEt, and then with AcOEt–acetone (1 : 1) gave **11c** (12.66 g, 48.5%) as an oil. IR (Nujol) ν: 3300, 1645 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 2.0—2.6 (1H, m), 2.92 (2H, d, *J* = 7 Hz), 4.05 (4H, d, *J* = 5 Hz), 5.7 (4H, br s), 7.2—8.2 (5H, m). MS *m/z* (relative intensity): 248 (M^+, 25), 173 (79), 145 (38), 119 (38), 105 (100), 104 (67).

5-Dimethylaminomethyl-6-oxo-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4,-*b*]pyridine (5a)——A mixture of **12c** (196 mg, 0.803 mmol), *p*-TsCl (600 mg, 3.15 mmol), and anhydrous pyridine (3 ml) was stirred at room temperature for 2 h. The mixture was quenched with H_2O and evaporated, and the residue (1.47 g) was purified by dry column chromatography on silica gel (30 g, CH_2Cl_2–Et_2O, 10 : 1) to afford 6-oxo-3-phenyl-5-*p*-toluenesulfonyloxymethyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (**12e**, 260 mg) as an oil. Me_2NH gas was passed through a solution of **12e** (260 mg, 0.653 mmol) in pyridine (3 ml) for 10 min under ice cooling. The mixture was stirred at room temperature overnight, and evapoated. Recrystallization of the crystalline residue from EtOH gave **5a** (152 mg, 70% from **12c**) as colorless plates, mp 154—160 °C (EtOH). IR (Nujol) ν: 1700, 1650 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.15 (6H, s), 2.3—3.2 (6H, m), 7.4—7.9 (5H, m). MS *m/z* (20 eV, relative intensity): 271 (M^+, 11), 226 (28), 168 (37), 105 (53), 59 (61), 58 (100). *Anal.* Calcd for $C_{15}H_{17}N_3O_2$: C, 66.40; H, 6.32; N, 15.49. Found: C, 66.51; H, 6.23; N, 15.32.

Compound **5b** was obtained as colorless plates in the same manner in 51% yield from **12d**, mp 151—153 °C ($EtOH$–H_2O). IR (Nujol) ν: 1700, 1640 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.10 (3H, d, *J* = 7 Hz), 2.15 (6H, s), 2.1—2.7 (4H, m), 3.27 (1H, q, *J* = 7 Hz), 7.3—7.9 (5H, m). MS *m/z* (20 eV, relative intensity): 285 (M^+, 0.7), 226 (5), 225 (19), 105 (6), 59 (7), 58 (100). *Anal.* Calcd for $C_{16}H_{19}N_3O_2$: C, 67.34; H, 6.71; N, 14.73. Found: C, 67.29; H, 6.60; N, 14.45.

***N*-Methyl-*N*-(3-phenyl-5-isoxazolyl)-1-methylnipecotamide (6a)**——A solution of EtMgBr, freshly prepared from Mg (0.85 g, 35.0 mmol) and EtBr (2.82 g, 25.9 mmol) in anhydrous THF (25 ml), was added dropwise to a solution of 5-methylamino-3-phenylisoxazole (**14b**, 6.10 g, 35.0 mmol) in anhydrous THF (35 ml) under ice cooling. The mixture was stirred at room temperature for 30 min, then ethyl 1-methylnipecotate (3.00 g, 17.5 mmol) in anhydrous THF (7 ml) was added and the whole was stirred at room temperature for 30 min, and heated under reflux for another 3 h. The reaction mixture was quenched with H_2O and evaporated. The residue was dissolved in Et_2O and filtered. The filtrate was extracted with 2 N HCl, and the aqueous layer was washed with Et_2O and basified with 7% NH_4OH. Extraction with Et_2O and evaporation of the solvent gave **6a** (3.87 g, 73.8% from ethyl 1-methylnipecotate) as an oil. IR (neat) ν: 1685, 1620, 1575 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.4—2.4 (6H, m), 2.27 (3H, s), 2.6—3.1 (3H, m), 3.42 (3H, s), 6.48 (1H, s), 7.3—7.9 (5H, m). MS *m/z* (relative intensity): 299 (M^+, 3), 282 (13), 126 (49), 124 (33), 98 (100), 97 (29), 70 (33), 58 (36).

Compound **6a** was converted into the fumarate in a usual manner to give **6a**·fumarate as colorless plates, mp 210—213 °C (CH_3COCH_3). *Anal.* Calcd for $C_{21}H_{25}N_3O_6$: C, 60.71; H, 6.07; N, 10.12. Found: C, 60.52; H, 6.09; N, 9.95.

Compound **14b** and ethyl 1-ethylnipecotate were treated in the same manner to give *N*-methyl-*N*-(3-phenyl-5-isoxazolyl)-1-ethylnipecotamide (**6b**, 81.2%) as an oil. MS *m/z* (relative intensity): 313 (M^+, 14), 298 (39), 140 (70), 138 (45), 112 (100), 84 (38), 72 (37). Compound **6b**·fumarate as colorless plates, mp 183—184 °C (CH_3COCH_3). *Anal.* Calcd for $C_{22}H_{27}N_3O_6$: C, 61.52; H, 6.34; N, 9.79. Found: C, 61.81; H, 6.10; N, 9.45.

Compound **14b** and ethyl 1-propylnipecotate were treated in the same manner to give *N*-methyl-*N*-(3-phenyl-5-isoxazolyl)-1-propylnipecotamide (**6c**, 57.2%) as an oil. MS *m/z* (relative intensity): 327 (M^+, 5), 298 (100), 157 (17),

155 (21), 144 (20), 126 (23). Compound **6c**·fumarate as colorless plates, 139—140 °C (CH_3COCH_3). *Anal.* Calcd for $C_{23}H_{29}N_3O_6$: C, 62.29; H, 6.59; N, 9.48. Found: C, 62.15; H, 6.71; N, 9.20.

Compound **14b** and ethyl 1-isopropylnipecotate were treated in the same manner to give *N*-methyl-*N*-(3-phenyl-5-isoxazolyl)-1-isopropylnipetamide (**6d**, 77.3%) as an oil. MS *m/z* (relative intensity): 327 (M^+, 14), 312 (100), 126 (23), 110 (36), 84 (31), 56 (26). Compound **6d**·fumarate as colorless plates, mp 144—146 °C (CH_3COCH_3). *Anal.* Calcd for $C_{23}H_{29}N_3O_6$: C, 62.29; H, 6.59; N, 9.48. Found: C, 62.33; H, 6.54; N, 9.60.

2-Ethylamino-*N*-(3-phenyl-5-isoxazolyl)acetamide (14d)——Chloroacetyl chloride (10.64 g, 94.2 mmol) was added to a mixture of 5-amino-3-phenylisoxazole (**14a**, 5.68 g, 35.5 mmol), Et_3N (8.93 g, 88.2 mmol), and CH_2Cl_2 (60 ml) under ice cooling and the mixture was stirred at room temperature overnight. The reaction mixture was quenched with H_2O under ice cooling, and washed with aqueous Na_2CO_3, and H_2O. The organic layer was dried over $MgSO_4$, and evaporation of the solvent gave crude 2-chloro-*N*-(3-phenyl-5-isoxazolyl)acetamide (**14c**, 12.14 g). A solution of $EtNH_2$ (9.5 g, 211 mmol) and Et_3N (21 g, 208 mmol) in $CHCl_3$ (35 g) was added to a suspension of **14c** (12.14 g) in $CHCl_3$ (130 ml) under ice cooling, and the mixture was stirred at room temperature overnight. The reaction mixture was evaporated and the residue was dissolved in 0.2 N HCl. The aqueous layer was washed with AcOEt, basified with aqueous Na_2CO_3, and extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$, and evaporated to give **14d** (6.16 g, 70.7% from **14a**) as an oil. IR (neat) ν: 3200, 1705 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.15 (3H, t, J=7 Hz), 2.73 (2H, q, J=7 Hz), 3.45 (2H, s), 4.8 (2H, br s), 6.70 (1H, s), 7.3—8.0 (5H, m).

5-[2-(*N*-*tert*-Butoxycarbonyl-*N*-ethyl)aminoethyl]amino-3-phenylisoxazole (14f)——A solution of **14d** (49.91 g, 204 mmol) in anhydrous THF (400 ml) was added dropwise to a suspension of $LiAlH_4$ (30 g, 791 mmol) in anhydrous THF (800 ml) under N_2 with ice cooling. The reaction mixture was stirred at room temperature for 6 h, then quenched with H_2O (100 ml) under ice cooling. The organic layer was dried over $MgSO_4$, and evaporated to afford 5-(2-ethylaminoethyl)amino-3-phenylisoxazole (**14e**, 43.74 g) as an oil. IR (neat) ν: 3280, 1620 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7 Hz), 2.2—3.0 (5H, m), 3.1—3.5 (2H, m), 5.25 (1H, s), 5.3 (1H, br s), 7.2—7.9 (5H, m). A solution of **14e** (43.74 g) in $CHCl_3$ (270 ml) was mixed with Et_3N (22.4 g, 221 mmol) and a solution of *tert*-butyl S-(4,6-dimethylpyrimidin-2-yl)thiocarbonate (50 g, 208 mmol) in $CHCl_3$ (110 mmol) at room temperature. The reaction mixture was stirred at room temperature for 5 h, then quenched with H_2O, and product isolation (CH_2Cl_2, $MgSO_4$) gave a residue (98 g), which was purified by column chromatography on silica gel (1 kg, CH_2Cl_2, then CH_2Cl_2–Et_2O, 10 : 1, and finally CH_2Cl_2–AcOEt, 10 : 1) to afford **14f** (42.42 g, 62.9% from **14d**) as an oil. IR (neat) ν: 3300, 1680, 1615 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7 Hz), 1.48 (9H, s), 3.25 (2H, q, J=7 Hz), 3.43 (4H, s), 5.25 (1H, s), 5.3 (1H, br s), 7.2—7.9 (5H, m). MS *m/z* (relative intensity): 331 (M^+, 0.2), 146 (43), 106 (36), 105 (38), 58 (88), 57 (100).

1-Ethyl-6-methyl-4-(3-phenyl-5-isoxazolyl)hexahydro-1*H*-1,4-diazepin-5-one (7)——A solution of freshly distilled methacryloyl chloride (60 g, 574 mmol) in $CHCl_3$ (200 ml) was added dropwise over 1 h under ice cooling to a solution of **14f** (47.29 g, 143 mmol) and Et_3N (150 g, 1.48 mol) in $CHCl_3$ (600 ml). The reaction mixture was stirred at room temperature overnight and quenched with H_2O. The organic layer was washed with aqueous Na_2CO_3, dried over $MgSO_4$, and evaporated to give crude *N*-[2-(*N'*-*tert*-butoxycarbonyl-*N'*-ethyl)aminoethyl]-2-methyl-*N*-(3-phenyl-5-isoxazolyl)-2-propenamide (**14g**, 104.26 g) as an oil. Compound **14g** (102.26 g) was dissolved in CF_3CO_2H (200 ml) under ice cooling and the reaction mixture was stirred at room temperature for 3 h. The mixture was evaporated, and the residue was dissolved in AcOEt, and washed with aqueous NaOH and H_2O. This solution was dried over $MgSO_4$ and evaporated to give crude *N*-(2-ethylaminoethyl)-2-methyl-*N*-(3-phenyl-5-isoxazolyl)-2-propenamide (**14h**, 71.60 g) as an oil. A mixture of **14h** (69.60 g) and AcOH (30 g) in PhH (1 l) was heated under reflux overnight. The reaction mixture was evaporated, and the residue was diluted with AcOEt, and extracted with 0.2 N HCl. The aqueous layer was basified with aqueous NaOH, and extracted with AcOEt, The organic layer was dried over $MgSO_4$, and evaporation of the solvent gave a residue (9.40 g), which was purified by column chromatography on silica gel (500 g, hexane–Et_2O–Et_3N, 2 : 1 : 1) to afford **7** (2.94 g, 7.3% from **14f**) as an oil. IR (neat) ν: 1690 cm^{-1}. ^1H-NMR ($CDCl_3$) δ: 1.10 (3H, t, J=7 Hz), 1.27 (3H, d, J=7 Hz), 1.9—3.5 (5H, m), 2.57 (2H, q, J=7 Hz), 3.5—4.8 (2H, m), 6.85 (1H, s), 7.3—7.9 (5H, m). MS *m/z* (relative intensity): 299 (M^+, 7), 195 (46), 127 (71), 103 (100), 71 (46). Compound **7**·HCl as colorless plates, mp 205—208 °C (MeOH). IR (Nujol) ν: 2400—2300, 1700 cm^{-1}. *Anal.* Calcd for $C_{17}H_{22}ClN_3O_2$: C, 60.80; H, 6.60; Cl, 10.56; N, 12.51. Found: C, 61.02; H, 6.65; Cl, 10.56; N, 12.70.

6-Oxo-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (12g)——A mixture of **12a** (2.00 g, 6.99 mmol) and K_2CO_3 (2.00 g, 14.5 mmol) in BuOH (30 ml) was heated under reflux for 18 h. The reaction mixture was evaporated, and the residue was diluted with H_2O and acidified with 1 N HCl. Product isolation (AcOEt, $MgSO_4$) gave a residue (1.33 g), which was purified by column chromatography on silica gel (160 g, CH_2Cl_2, then CH_2Cl_2–AcOEt, 9 : 1) to afford **12g** (0.84 g, 56.1%) as colorless plates, mp 216—218 °C (THF). IR (Nujol) ν: 1690, 1660 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.5—3.1 (4H, m), 3.4 (1H, br s), 7.4—7.9 (5H, m). MS *m/z* (20 eV, relative intensity): 214 (M^+, 75), 170 (15), 169 (79), 143 (26), 105 (100). *Anal.* Calcd for $C_{12}H_{10}N_2O_2$: C, 67.28; H, 4.71; N, 13.08. Found: C, 67.08; H, 4.51; N, 12.92.

3-Phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (13d)——$LiAlH_4$ (100 mg, 2.64 mmol) was added in portions to a solution of **12g** (200 mg, 0.935 mmol) in anhydrous THF (10 ml) under ice cooling, and the reaction mixture was stirred at room temperature for 3 h. The mixture was quenched with H_2O, with cooling, and product isolation

(AcOEt, $MgSO_4$) gave a residue (174 mg), which was purified by silica gel thin layer chromatography (PhMe–Et_2O, 3 : 1) to afford **13d** (113 mg, 60.4%) as colorless plates, mp 112—115 °C (hexane–PhH). IR (Nujol) ν: 3200, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.2 (2H, m), 2.67 (2H, t, J=6 Hz), 3.2—3.6 (2H, m), 4.6 (1H, br s), 7.3—7.9 (5H, m). MS *m/z* (20 eV, relative intensity): 200 (M^+, 100), 199 (24), 105 (37), 69 (18), 68 (19). *Anal.* Calcd for $C_{12}H_{12}N_2O$: C,

	R^1	NR^2R^3	R^4
1a	Me	NEt_2	H
1b	H	NMe_2	H
1c	Me	N(Et)Pr	H
1d	Me	NPr_2	H
1e	Me	NEt_2	Et
1f	Me	NEt_2	iso-Pr
1g	Pr	NEt_2	H

	R^1	R^2	R^3	R^4
2a	H	Me	H	H
2b	CH_2Ph	Me	H	H
2c	CH_2Ph	H	H	Me
2d	H	H	Me	H
2e	CH_2Ph	H	Me	H

Chart 10

	R^1	R^2	R^3
15a	H	H	Et
15b	H	H	iso-Pr
15c	Me	H	Et
15d	Me	H	iso-Pr
15e	Me	COC(Me)=CH_2	Et
15f	Me	COC(Me)=CH_2	iso-Pr

Chart 11

Table VI. 4-Substituted 5-Amino-3-phenylisoxazoles (**15a**—**f**, **1e**, **f**)

Compd. No.	R^1	R^2	R^3	Yield	mp (°C)	Recrystn. solvent	IR (cm^{-1})	MS *m/z* (M^+)
15a	H	H	Et	37[a)]	Oil	—	[b)]	188
15b	H	H	iso-Pr	37[a)]	Oil	—	1646, 1635 (neat)	202
15c	Me	H	Et	60[c)]	103—105[h)]	PhMe	[b)]	202
15d	Me	H	iso-Pr	50[c)]	102—103[i)]	Hexane–AcOEt	[b)]	216
15e	Me	COC(Me)=CH_2	Et	86	Oil	—	1675, 1635 (neat)	270
15f	Me	COC(Me)=CH_2	iso-Pr	77	Oil	—	[b)]	284
1e	Me	COCH(Me)CH_2NEt_2	Et	28[d)]	92—96[e,j)]	CH_3COCH_3	1685, 1640 (neat)	343
1f	Me	COCH(Me)CH_2NEt_2	iso-Pr	25[d)]	119—120[e,k)]	CH_3COCH_3	1690, 1635 (neat)	357

a) Yield from benzonitrile. *b*) Not determined. *c*) Yield from **15a**, or **15b**, respectively. *d*) Yield of the free base. *e*) Fumarate. *f*) **15a**: ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, J=7 Hz), 2.32 (2H, q, J=7 Hz), 4.60 (2H, br s), 7.2—7.7 (5H, m). **15b**: ^{1}H-NMR ($CDCl_3$) δ: 1.20 (6H, d, J=7 Hz), 2.80 (1H, quintet, J=7 Hz), 4.33 (2H, br s), 7.46 (5H, s). **15c**: ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J=7 Hz), 2.32 (2H, q, J=7 Hz), 3.07 (3H, d, J=6 Hz), 4.20 (1H, br s), 7.2—7.7 (5H, m). **15d**: ^{1}H-NMR ($CDCl_3$) δ: 1.15 (6H, d, J=7 Hz), 2.75 (1H, quintet, J=7 Hz), 3.05 (3H, d, J=6 Hz), 4.05 (1H, br s), 7.42 (5H, s). **15e**: ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, t, J=7 Hz), 1.92 (3H, t, J=1.5 Hz), 2.47 (2H, q, J=7 Hz), 3.32 (3H, s), 5.17 (2H, t, J=1.5 Hz), 7.2—7.8 (5H, m). **15f**: ^{1}H-NMR ($CDCl_3$) δ: 1.10 (6H, d, J=7 Hz), 1.93 (3H, br s), 2.82 (1H, quintet, J=7 Hz), 3.30 (3H, s), 5.23 (2H, br s), 7.45 (5H, s). *g*) **1e**·fumarate: *Anal.* Calcd for $C_{24}H_{33}N_3O_6$: C, 62.72; H, 7.24; N, 9.14. Found: C, 62.50; H, 7.22; N, 8.87. **1f**·fumarate: *Anal.* Calcd for $C_{25}H_{35}N_3O_6$: C, 63.40; H, 7.45; N, 8.87. Found: C, 63.53; H, 7.56; N, 8.59. *h*) Obtained as colorless plates. *i*) Obtained as colorless plates. *j*) Obtained as colorless plates. *k*) Obtained as colorless plates.

71.98; H, 6.04; N, 13.99. Found: C, 72.00; H, 6.07; N, 13.70.

7-(2-Methyl-2-propenoyl)-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (13e)——A solution of methacryloyl chloride (0.71 ml, 7.27 mmol) in $CHCl_3$ (3 ml) was added dropwise to a mixture of **13d** (960 mg, 4.80 mmol) and Et_3N (1.00 ml, 7.17 mmol) in $CHCl_3$ (15 ml) under ice cooling. The mixture was stirred at room temperature for 15 h, then Et_3N (1.00 ml, 7.17 mmol) and methacryloyl chloride (0.71 ml, 7.27 mmol) were added and the stirring was continued for another 4 h. The product was isolated ($CHCl_3$, $MgSO_4$) and the residue (2.74 g) was purified by column chromatography on silica gel (200 g, $CHCl_3$–AcOEt, 50:1) to afford **13e** (848 mg, 65.9%) as colorless plates, mp 106—107 °C. IR (KBr) ν: 1660, 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.1 (5H, br s), 2.75 (2H, t, J=7 Hz), 3.7—4.0 (2H, m), 5.32 (2H, d, J=7 Hz), 7.3—7.9 (5H, m).

7-(3-Diethylamino-2-methylpropanoyl)-3-phenyl-4,5,6,7-tetrahydroisoxazolo[5,4-*b*]pyridine (8)——A mixture of **13e** (800 mg, 2.98 mmol), Et_2NH (20 ml), and AcOH (0.1 ml) was heated under reflux for 17 h. The reaction mixture was evaporated, and the residue was dissolved in PhMe, and extracted with 0.4 N HCl. The aqueous layer was washed with PhMe, basified with conc. NH_4OH to pH 9, and extracted with Et_2O. The organic layer was washed with H_2O, dried over $MgSO_4$, and evaporated to give **8** (964 mg, 94.7%) as colorless plates, mp 55—57 °C. IR (Nujol) ν: 1685, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (6H, t, J=7 Hz), 1.22 (3H, d, J=7 Hz), 1.7—3.0 (6H, m), 2.48 (4H, q, J=7 Hz), 3.4—4.0 (2H, m), 4.0—4.5 (1H, m), 7.3—7.9 (5H, m). MS m/z (relative intensity): 341 (M^+, 3), 105 (7), 87 (13), 86 (100), 58 (10). M_r (high-resolution MS). Calcd for $C_{20}H_{27}N_3O_2$: 341.2102. Found: 341.2090.

The syntheses of **1a**—**d**, **1g**, and **2a**—**e** were previously described.[1a,b]

3-Diethylamino-2,*N*-dimethyl-*N*-(4-ethyl-3-phenyl-5-isoxazolyl)propanamide (**1e**) and 3-diethylamino-2,*N*-dimethyl-*N*-(4-isopropyl-3-phenyl-5-isoxazolyl)propanamide (**1f**) were prepared in the same manner as described previously,[1b] from 5-amino-4-ethyl-3-phenylisoxazole (**15a**) and 5-amino-4-isopropyl-3-phenylisoxazole (**15b**), respectively, *via* compounds **15c**—**f**. The yields, physical constants, selected spectroscopic data, and the results of elemental analysis of **15a**—**f**, and of the free bases or fumarates of **1e**, **f** are listed in Table VI.

Pharmacology

Effects on the Conditioned-Avoidance Response in Rats——An automatic shuttle box designed in our laboratories was used as the testing apparatus. The conditioned stimuli (60 W light bulbs) were presented for 10 s, and an unconditioned stimulus (a scrambled electric shock of 100 V) was delivered to the grid floor for 3 s from 7 s after the onset of the conditioned stimuli. The intertrial interval was 60 s. Well trained and conditioned rats were given intraperitoneal doses of the test compounds. Three to six animals were used for each treatment. At 0.5, 1, 2, 4, 6, and 8 h after the administration, the response of each animal to the light was determined by 10 successive trials. The ED_{50} was defined as the dose suppressing the mean conditioned rate by 50% at the time of peak compound activity.

Other pharmacological procedures described in this report were carried out in the manner previously reported.[1]

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Synthesis and Activity of Optical Isomers of Nipradilol[1]

MASAMI SHIRATSUCHI,*,[a] KIYOSHI KAWAMURA,[a] TOSHIHIRO AKASHI,[a]
HIROSHI ISHIHAMA,[a] MASAKI NAKAMURA,[a]
and FUMIO TAKENAKA,[b]

Tokyo Research Laboratories, Kowa Company, Ltd.,[a] Noguchi-cho, Higashimurayama, Tokyo 189, Japan and Kumamoto Women's University,[b] Kengun-cho, Kumamoto 862, Japan

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Four optical isomers of nipradilol (NIP) were prepared from (3*R*)- or (3*S*)-3,4-dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran by glycidylation and amination, and evaluated for β-blocking and vasodilating activities. The order of potency of β-blocking activity was *S*,*R*-NIP ≫ *S*,*S*-NIP > *R*,*R*-NIP ≫ *R*,*S*-NIP. As regards vasodilating activity, *S*,*R*-NIP and *R*,*R*-NIP were more potent than *S*,*S*-NIP and *R*,*S*-NIP.

Keywords——nipradilol; synthesis; optical isomer; β-blocking activity; α-blocking activity; vasodilating activity; benzopyran

Nipradilol (NIP) is a synthetic drug having β-blocking and vasodilating activities (direct vasodilating activity and α-blocking acitivitiy),[2] and is used to treat cardiovascular diseases.

OH
1′ 2′ 3′
OCH_2CHCH_2NH iso-Pr
O
ONO_2
NIP

Since NIP has asymmetric carbon atoms at the 3-position of the benzopyran ring and the 2′-position of the side chain, four optical isomers can exist theoretically. The *N*,*O*-bis-L-menthoxyacetyl derivatives show four peaks on high-performance liquid chromatography (HPLC), and the areas of the peaks are nearly equal (Fig. 1). We reported the resolution of NIP in this journal previously.[3] The result of further studies on the optical isomers of NIP and their pharmacological activities are presented here.

Chemistry

The starting compounds (3*R*)-3,4-dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (**4**) and (3*S*)-3,4-dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (**5**), were obtained by optical resolution of 3,4-dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (**1**) (Chart 1).[2] Esterification was carried out by the action of (L)-*N*-mesylphenylalanine on **1** in the presence of a condensing agent (*e.g.*, dicyclohexylcarbodiimide). (3*R*)-3,4-Dihydro-8-(*N*-mesylphenylalanyloxy)-3-nitroxy-2*H*-1-benzopyran (**2**), whose specific rotation was −17.2°, was separated from the resulting mixture of crystalline and oily diastereomers by recrystallization. The C_3-position was determined to have *R* configuration by X-ray analysis.[4] Hydrolysis of the optically active (3*R*) ester **2** gave the (3*R*)-8-hydroxy compound **4**. Similarly, the (3*S*) ester **3**, whose specific rotation was +17.2°, was produced by using (D)-*N*-mesylphenylalanine instead of (L)-*N*-mesylphenylalanine, and hydrolysis of **3** gave the (3*S*)-8-hydroxy compound

(L)-PhCH$_2$CH(NHMs)COOH recryst. → 2 (OCOCH(NHMs)CH$_2$Ph, (R) ONO$_2$) NaOH → 4 (OH, (R) ONO$_2$)

1 (OH, ONO$_2$)

(D)-PhCH$_2$CH(NHMs)COOH recryst. → 3 (OCOCH(NHMs)CH$_2$Ph, (S) ONO$_2$) NaOH → 5 (OH, (S) ONO$_2$)

Chart 1

1 + ClCH$_2$CH—CH$_2$ (epoxide) K_2CO_3 → OCH$_2$CH—CH$_2$ (epoxide), ONO$_2$ iso-PrNH$_2$ → OCH$_2$CH(OH)CH$_2$NHiso-Pr, ONO$_2$

1 | 7 : R, 7′: S | 6: 2′S, 8: 2′R | 9: 2′S-NIP, 10: 2′R-NIP

Chart 2

5. Then, we examined the reaction of **1** with epichlorohydrin to synthesize the glycidyl ether **6** (Chart 2).

In the reaction of (*R*)- and/or (*S*)-epichlorohydrin with phenols, Baldwin *et al.*[5-7] obtained mainly methyloxiranes whose configuration at the C_2-position in the side chain was inverted. On the basis of this result, it was supposed that (2′*S*)-3,4-dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (**6**) and (2′*R*)-3,4-dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (**8**) could be obtained as major products from (*R*)-epichlorohydrin (**7**) and (*S*)-epichlorohydrin (**7′**) respectively. The amination of **6** gave (2′*S*)-3,4-dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (2′*S*-NIP) (**9**) and that of **8** gave (2′*R*)-3,4-dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (2′*R*-NIP) (**10**) (Fig. 1). On the other hands, the glycidylation of **1** with (2*S*)-3-(2-nitrobenzenesulfonyloxy)-1,2-epoxypropane (**11**) and subsequent amination with isopropylamine gave stereoselectively 2′*S*-NIP **9** (Chart 3). Therefore it was supposed that this substitution reaction proceeds with retention of configuration. We synthesized four optical isomers of NIP by utilizing **11** and its optical isomer, (2*R*)-3-(2-nitrobenzenesulfonyloxy)-1,2-epoxypropane (**12**). Thus, the glycidylation of **4** with **11** and subsequent amination with isopropylamine gave (2′*S*), (3*R*)-3,4-dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (*S*,*R*-NIP) (**13**) (Chart 4). The *N*,*O*-

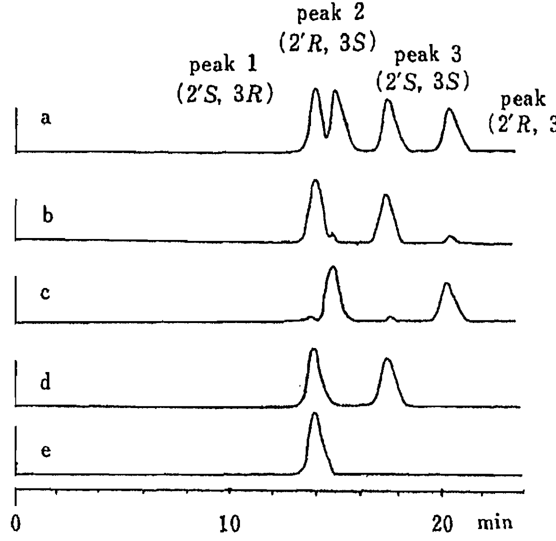

Fig. 1. Chromatogram of *N*,*O*-Bis-L-methoxyacetyl Derivatives of (a) NIP, (b) 2'*S*-NIP Obtained from (*R*)-Epichlorohydrin, (c) 2'*R*-NIP Obtained from (*S*)-Epichlorohydrin, (d) 2'*S*-NIP Obtained from **11**, (e) *S*,*R*-NIP

Column: Partisil-10 (10 μm) (Whatman), 4×200 mm. Eluent: hexane–ethyl acetate (5 : 1), 1.5 ml/min. Detection: UV 275 nm.

Chart 3

Chart 4

bis-L-methoxyacetyl derivative of **13** gave a single peak on HPLC (Fig. 1). The other isomers of NIP were similarly synthesized, and each gave a single peak on HPLC (Table I). These products were identified by comparison of the circular dichroism spectra and nuclear magnetic resonance (NMR) spectra with those of compounds of known configuration.[3]

Biological Activity and Discussion

***β*-Blocking Activity**——The compounds listed in Table I were tested for antagonistic action towards isoproterenol, *i.e.* *β*-blocking action, using isolated atrium and trachea of guinea pigs. The results are shown in Tables II and III. In the tables, pA_2 is the reciprocal logarithm of the molar concentration of each test compound required to shift the dose–response curve of isoproterenol in parallel to 2-fold higher dose. The parenthesized figures are efficacy ratios based on NIP. The results demonstrate that the *β*-blocking activity of *R*,*R*-NIP is about one-tenth of that of NIP, while that of *S*,*R*-NIP is about 3 to 8 times as strong as

TABLE I. Optical Isomers of NIP

Compd. No.	Structure	Yield[a] (%)	mp (°C)	$[\alpha]_D^{25}$ (°)
13	(S,R-NIP)	74.4	105.3—105.8	+16.2
14	(S,S-NIP)	63.1	140.5—141.0	−15.8
15	(R,R-NIP)	70.3	140.3—141.0	+15.5
16	(R,S-NIP)	71.1	105.3—106.1	−16.7

a) Overall yield of glycidylation and amination.

TABLE II. Effects of NIP and Its Isomers on β-Adrenoceptors in Guinea-pig

Antagonist	Right atrium (β_1-Adrenoceptors)			Left atrium (β_1-Adrenoceptors)		
	n[a]	$pA_2 \pm$ S.E. (Ratio)	Slope ± S.E.	n[a]	$pA_2 \pm$ S.E. (Ratio)	Slope ± S.E.
NIP	5	9.07±0.12 (1.00)	−0.98±0.05	5	8.72±0.10 (1.00)	−1.17±0.04
S,R-NIP	5	9.56±0.08 (3.09)	−0.99±0.03	5	9.60±0.12 (7.59)	−1.05±0.03
S,S-NIP	5	8.14±0.20 (0.12)	−1.08±0.06	5	8.17±0.09 (0.28)	−1.04±0.06
R,R-NIP	6	7.64±0.15 (0.037)	−0.91±0.05	5	7.65±0.11 (0.085)	−0.93±0.06
R,S-NIP	5	5.83±0.07[b] (0.0006)	—	6	5.48±0.10[b] (0.0006)	—
Propranolol	3	8.70±0.11 (0.43)	−0.99±0.01	4	8.57±0.17 (0.71)	−0.99±0.07

pA_2 and slope were calculated as described by Arunlakshana and Schild.[8] The agonist used was isoprotenol. a) Number of guinea-pigs. b) pA_2 estimated from the shift produced in the presence of 10^{-5} M antagonist.[9]

that of NIP, and that of *S,S*-NIP is about 3 times as strong as that of *R,R*-NIP. Previous reports have shown that β-blockers possessing the *S* configuration are more potent than those of *R* configuration among compounds of the aryloxypropanolamine type [propranolol, practolol and timolol].[10-13] This finding is in accordance with our results. Furthermore, the β-blocking activity of *S,R*-NIP is about 26 times as strong as that of *S,S*-NIP. Therefore, it

Table III. Effects of NIP and Its Isomers on β-Adrenoceptors in Guinea-pig

Antagonist	Tracheal strip (β_2-Adrenoceptors)		
	n	$pA_2 \pm$ S.E. (Ratio)	Slope $\pm$ S.E.
NIP	6	8.49 ± 0.11 (1.00)	-0.95 ± 0.05
S,R-NIP	6	9.18 ± 0.15 (4.90)	-1.09 ± 0.11
S,S-NIP	6	7.60 ± 0.09 (0.13)	-0.95 ± 0.06
R,R-NIP	6	7.39 ± 0.23 (0.079)	-0.85 ± 0.09
R,S-NIP	6	5.84 ± 0.11 (0.002)	-0.80 ± 0.06
Propranolol		—	—

Table IV. Effects of NIP and Its Isomers on K^+-Induced Contracture and NE Induced Contraction in Canine Mesenteric Arteries

	K^+-Contracture		Norepinephrine contraction (α-Adrenoceptors)		
	n	$pD_2 \pm$ S.E. (Ratio)	n	$pA_2 \pm$ S.E. (Ratio)	Slope $\pm$ S.E.
NIP	7	6.17 ± 0.07 (1.00)	6	6.81 ± 0.09 (1.00)	-0.85 ± 0.07
S,R-NIP	7	6.24 ± 0.07 (1.17)	6	7.12 ± 0.10 (2.04)	-0.94 ± 0.07
R,R-NIP	6	6.35 ± 0.03 (1.51)	6	6.75 ± 0.12 (0.87)	-0.90 ± 0.09
S,S-NIP	7	4.69 ± 0.15 (0.03)	6	5.65 ± 0.12 (0.07)	-0.66 ± 0.06
R,S-NIP	7	4.77 ± 0.10 (0.04)	6	5.44 ± 0.06 (0.04)	-0.78 ± 0.03

pD_2 = concentration of antagonist required to produce an agonist dose/ratio of 2. pA_2 and slope were calculated as described by Arunlakshana and Schild.[8]

is suggested that the 3*R* configuration (at the nitroxy moiety) is superior to the 3*S* configuration with regard to approach to the receptor.

Vasodilating activity: The compounds indicated in Table I were tested for antagonistic action on potassium contracture (direct vasorelaxing activity) and their antagonistic action on the contractile activity of norepinephrine (NE) (α-blocking activity) in the isolated superior mesentric artery of dogs. The results are shown in Table IV. In the table, pD_2 represents the reciprocal logarithm of the molar concentration of each test compound required to inhibit the maximum reaction to potassium (25 mM K^+) by 50%. The parenthesized figures have the same meanings as in Table II. The above results demonstrate that the vasodilating activity of *R,R*-NIP is nearly equivalent to that of NIP, and the vasodilating activity of *S,R*-NIP is about 1.1 to 2 times that of NIP, while the vasodilating activities of *S,S*-NIP and *R,S*-NIP are weaker than that of NIP. Therefore, it is suggested that the 3*R* configuration at the nitroxy

moiety has higher pharmacological activity than the 3*S* configuration.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were measured with JASCO IRA-1 and Shimadzu IR-435 spectrometers. NMR spectra were taken at 60 MHz with a Varian EM-360 spectrometer and at 100 MHz with a JEOL JNM-MH-100 spectrometer. Chemical shifts are expressed in δ (ppm) values. The following abbreviations are used: s = singlet, d = doublet, q = quartet, dd = double doublet, m = multiplet and br = broad. Mass spectra (MS) were measured with JEOL JMS-D-300 and JMS-D-100 mass spectrometers.

(3*R*)-3,4-Dihydro-8-(*N*-mesyl-L-phenylalanyloxy)-3-nitroxy-2*H*-1-benzopyran (2)——Compound **1** (31.8 g, 150 mmol) was dissolved in CH_2Cl_2 (500 ml) and *N*-mesyl-L-phenylalanine (36.6 g, 150 mmol) and 4-dimethylamino-pyridine (DMAP) (3.7 g, 30 mmol) were added. A solution of dicyclohexylcarbodiimide (40 g, 194 mmol) in CH_2Cl_2 (130 ml) was added with stirring at room temperature, and the mixture was stirred for 12 h at the same temperature. The precipitate was removed from the reaction mixture by filtration. The filtrate was concentrated, and the residue was dissolved in AcOEt. This solution was washed successively with 5% HCl, 5% aqueous NaOH and H_2O, then evaporated, and the residue was dissolved in tetrahydrofuran (THF). The insoluble materials were removed by filtration. Hexane was added, and the solution was left to stand. The precipitated crystals were collected by filtration, and recrystallized from acetone to give 12.8 g (yield, 19.4%) of the desired product as colorless prisms, mp 191—195 °C (dec.), $[\alpha]_D^{25}-17.2°$ (c=3, THF). NMR ($CDCl_3$) δ: 2.74 (3H, s, CH_3), 3.08—3.50 (4H, m, C_4-H, CH_2-Ar), 4.24—4.36 (2H, m, C_2-H), 4.60—4.80 (1H, m, >C<u>H</u>NH), 4.92 (1H, d, J=9 Hz, NH), 5.36—5.56 (1H, m, C_3-H), 6.80—7.10 (3H, m, Ar-H), 7.32 (5H, s, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3240 (NH), 1778 (COO), 1635, 1272 (ONO_2), 1325, 1150 (SO_2NH). MS m/z 436 (M^+). *Anal.* Calcd for $C_{19}H_{20}N_2O_8S$: C, 52.29; H, 4.62; N, 6.42. Found: C, 52.51; H, 4.74; N, 6.27.

(3*S*)-3,4-Dihydro-8-(*N*-mesyl-L-phenylalanyloxy)-3-nitroxy-2*H*-1-benzopyran (3)——In the same manner as described for **2**, crude **3** was prepared from **1** (31.8 g, 150 mmol), *N*-mesyl-D-phenylalanine (36.6 g, 150 mmol) and DMAP (3.7 g, 30 mmol). Recrystallization of crude **3** from acetone gave 11.2 g (yield, 17.0%) of the pure product as colorless prisms, mp 192—195 °C (dec.), $[\alpha]_D^{25}+17.2°$ (c=3, THF). The NMR spectrum and IR spectrum of **3** were identical with those of **2**.

(3*R*)-3,4-Dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (4)——Compound **2** (21.6 g, 49.5 mmol) was dissolved in THF (200 ml) at room temperature and MeOH (100 ml) and a 10% aqueous solution (40 ml) of NaOH were added. The mixture was stirred for 1 h, then adjusted to a pH of about 4 with cold HCl, and concentrated. The residue was taken up in $CHCl_3$, the pH of the aqueous layer was adjusted to pH 2 with dilute HCl, and the $CHCl_3$ layer was separated. The organic layer was washed with an aqueous solution of $NaHCO_3$ and also with H_2O, and the solvent was evaporated. Recrystallization of the residue from AcOEt–hexane gave 9.2 g (yield, 88.0%) of the desired product as colorless prisms, mp 129.0—130.5 °C, $[\alpha]_D^{25}+40.8°$ (c=3, $CHCl_3$). NMR ($CDCl_3$) δ: 2.84—3.42 (2H, m, C_4-H), 4.12—4.52 (2H, m, C_2-H), 5.32—5.50 (1H, m, C_3-H), 5.44 (1H, s, OH), 6.48—6.90 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3330 (OH), 1620, 1290 (ONO_2). *Anal.* Calcd for $C_9H_9NO_5$: C, 51.19; H, 4.30; N, 6.63. Found: C, 51.13; H, 4.30; N, 6.56.

(3*S*)-3,4-Dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (5)——In the same manner as described for **4**, crude **5** was prepared from **3** (21.6 g, 49.5 mmol) and a 10% aqueous solution (40 ml) of NaOH. Recrystallization of crude **5** from AcOEt–hexane gave 9.0 g (yield, 86.3%) of the pure product as colorless prisms, mp 129.0—130.5 °C, $[\alpha]_D^{25}-41.3°$ (c=3, $CHCl_3$). The NMR spectrum and IR spectrum of **5** were identical with those of **4**.

(2*R*)-3-(2-Nitrobenzenesulfonyloxy)-1,2-epoxypropane (12)——(*R*)-Glycerol acetonide[14] [bp 88—106 °C (18 mmHg), $[\alpha]_D^{25}-12.10°$ (c=5.64, MeOH)] (8.6 g, 65 mmol) was dissolved in $CHCl_3$ (70 ml), and Et_3N (10.0 g, 99 mmol) was added. A solution of tosyl chloride (13.8 g, 72 mmol) in $CHCl_3$ (30 ml) was added dropwise with stirring and ice cooling and the whole was stirred for 12 h, then washed with an aqueous solution of $KHCO_3$ and H_2O. The solvent was evaporated off, the residue (18.7 g) was dissolved in acetone (75 ml), and 1 N HCl (225 ml) was added. The mixture was stirred at 80 °C for 30 min, then concentrated and extracted with AcOEt (450 ml). The extract was washed with H_2O, and the solvent was evaporated off. The residue was purified by silica gel column chromatography [solvent: $CHCl_3$–MeOH (40:1)] to give 10.86 g (yield, 67.8%) of (*S*)-3-tosyloxy-1,2-propanediol as colorless needles, mp 57—60 °C, $[\alpha]_D^{25}+9.66°$ (c=7.35, MeOH). NMR ($CDCl_3$) δ: 2.40 (3H, s, CH_3), 3.68—4.08 (5H, m, HO-C<u>H</u>$_2$-C<u>H</u>-C<u>H</u>$_2$-O-), 7.25 (2H, d, J=7.5 Hz, Ar-H), 7.70 (2H, d, J=7.5 Hz, Ar-H). (*S*)-3-Tosyloxy-1,2-propanediol (14.90 g, 60.5 mmol) was dissolved in THF (140 ml), and MeONa (6.49 g, 120 mmol) was added with ice cooling. The mixture was stirred for 2 h. Then Et_3N (27.8 ml, 300 mmol) was added, and a solution of 2-nitrobenzenesulfonyl chloride (66.5 g, 300 mmol) in THF (150 ml) was added dropwise. The reaction mixture was stirred for 12 h, then filtered, and the filtrate was concentrated. The residue was purified by silica gel column chromatography [solvent: benzene–$CHCl_3$ (2:1)] to give 7.31 g (yield, 46.6%) of the desired compound as a pale yellow viscous oil, $[\alpha]_D^{25}+2.8°$ (c=15.2, $CHCl_3$), NMR ($CDCl_3$) δ: 2.60—2.90 (2H, m, -CH(-O-)C<u>H</u>$_2$), 3.16—3.36 (1H,

m, $\mathrm{-C\underline{H}\overset{O}{-}CH_2}$), 4.00—4.66 (2H, m, -O-CH$_2$-S-), 7.62—8.16 (4H, m, Ar-H). IR ν_{max}^{film} cm^{-1}: 3000 ($\underset{O}{\nabla}$), 1365, 1180 (SO$_2$). *Anal.* Calcd for $C_9H_9NO_6S$: C, 41.70; H, 3.50; N, 5.40. Found: C, 41.66; H, 3.52; N, 5.18.

(2*S*)-3-(2-Nitrobenzenesulfonyloxy)-1,2-epoxypropane (11)——(*R*)-3-Tosyloxy-1,2-propanediol [mp 60—62 °C, $[\alpha]_D^{24}$ −9.25° (c=2.12, MeOH)] (24.4 g, 99 mmol) was dissolved in a mixture of MeOH (30 ml) and Et$_2$O (15 ml). With ice cooling and stirring, MeONa (6.5 g, 120 mmol) was added in four portions at intervals of 1 h. The insoluble materials were then removed by filtration from the reaction mixture, and the solvent was evaporated at below 30 °C to give the crude (*R*)-glycidol (8.4 g). The crude product (8.4 g, 99 mmol) was dissolved in THF (120 ml), and Et$_3$N (11.1 g, 109 mmol) was added. Then, 2-nitrobenzenesulfonyl chloride (24.2 g, 109 mmol) was added with cooling and stirring, and the mixture was stirred for 2 h. The insoluble materials were removed from the reaction mixture by filtration, and the solvent was evaporated off. The residue was purified by silica gel column chromatography [solvent: benzene-CHCl$_3$ (2:3)] to give 15.45 g (yield, 60.1%) of the desired product. $[\alpha]_D^{25}$ −2.8° (c=10.0, CHCl$_3$). NMR (CDCl$_3$) δ: 2.60—2.92 (2H, m, $\mathrm{-CH\overset{O}{-}C\underline{H}_2}$) 3.16—3.36 (1H, m, $\mathrm{-C\underline{H}\overset{O}{-}CH_2}$), 4.00—4.66 (2H, m, -C$\underline{H}_2$-O-S-), 7.60—8.16 (4H, m, Ar-H). IR ν_{max}^{film} cm^{-1}: 3000 ($\underset{O}{\nabla}$), 1365, 1180 (SO$_2$). *Anal.* Calcd for $C_9H_9NO_6S$: C, 41.70; H, 3.50; N, 5.40. Found: C, 41.76; H, 3.51; N, 5.29.

(2'*R*),(3*R*)-3,4-Dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (18)——Compound **4** (3.75 g, 17.7 mmol) was dissolved in anhydrous THF (30 ml), and a suspension of 50% NaH (0.85 g, 17.7 mmol) in THF (9 ml) was added with ice cooling. The mixture was stirred for 15 min. A solution of **12** (4.6 g, 17.7 mmol) in THF (25 ml) was added, and the whole was stirred at a bath temperature of 70—75 °C for 1.5 h. The precipitate was removed by filtration, and the solvent was evaporated off. The residue was dissolved in CHCl$_3$ (50 ml), and this solution was washed with 5% aqueous NaOH and then with H$_2$O. The solvent was evaporated off. Recrystallization of the residue from acetone-MeOH gave 3.89 g (yield, 82.0%) of the desired product as colorless needles, mp 141.0—142.5 °C, $[\alpha]_D^{25}$ +34.0° (c=2, CHCl$_3$). NMR (CDCl$_3$) δ: 2.64—2.92 (2H, m, $\mathrm{-CH\overset{O}{-}C\underline{H}_2}$), 2.96—3.44 (3H, m, C$_4$, $\mathrm{-C\underline{H}\overset{O}{-}CH_2}$), 3.84—4.52 (4H, m, C$_2$-H, -OCH$_2$CH), 5.26—5.48 (1H, m, C$_3$-H), 6.52—6.88 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1280 (ONO$_2$). *Anal.* Calcd for $C_{12}H_{13}NO_6$: C, 53.93; H, 4.90; N, 5.24. Found: C, 53.70; H, 4.82; N, 5.13.

(2'*S*),(3*R*)-3,4-Dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (17)——In the same manner as described for **18**, crude **17** was prepared from **4** (3.75 g, 17.7 mmol) and **11** (4.6 g, 17.7 mmol). Recrystallization of crude **17** from acetone-MeOH gave 4.3 g (yield, 90.6%) of the pure product as colorless needles, mp 131.5—132.0 °C, $[\alpha]_D^{25}$ +25.3° (c=2, CHCl$_3$). NMR (CDCl$_3$) δ: 2.68—2.96 (2H, m, $\mathrm{-CH\overset{O}{-}C\underline{H}_2}$), 3.00—3.44 (3H, m, C$_4$-H, $\mathrm{-C\underline{H}\overset{O}{-}CH_2}$), 3.92—4.52 (4H, m, C$_2$-H, -OCH$_2$CH), 5.28—5.52 (1H, m, C$_3$-H), 6.57—6.92 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1280 (ONO$_2$). *Anal.* Calcd for $C_{12}H_{13}NO_6$: C, 53.93; H, 4.90; N, 5.24. Found: C, 53.71; H, 4.85; N, 5.12.

(2'*S*),(3*S*)-3,4-Dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (19)——In the same manner as described for **18**, crude **19** was prepared from **5** (3.75 g, 17.7 mmol) and **11** (4.6 g, 17.7 mmol). Recrystallization of crude **19** from acetone-MeOH gave 4.0 g (yield, 85.7%) of the pure product as colorless needles, mp 141.0—142.5 °C, $[\alpha]_D^{25}$ −32.8° (c=2, CHCl$_3$). NMR (CDCl$_3$) δ: 2.64—2.92 (2H, m, $\mathrm{-CH\overset{O}{-}C\underline{H}_2}$) 2.96—3.44 (3H, m, C$_4$-H, $\mathrm{-C\underline{H}\overset{O}{-}CH_2}$), 3.84—4.52 (4H, m, C$_2$-H, -OCH$_2$-CH), 5.26—5.48 (1H, m, C$_3$-H), 6.52—6.88 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1280 (ONO$_2$). *Anal.* Calcd for $C_{12}H_{13}NO_6$: C, 53.93; H, 4.90; N, 5.24. Found: C, 53.76; H, 4.90; N, 5.12.

(2'*R*),(3*S*)-3,4-Dihydro-8-(2,3-epoxypropoxy)-3-nitroxy-2*H*-1-benzopyran (20)——In the same manner as described for **18**, crude **20** was prepared from **5** (3.75 g, 17.7 mmol) and **12** (4.6 g, 17.7 mmol). Recrystallization of crude **20** from acetone-MeOH gave 4.1 g (yield, 86.0%) of the pure product as colorless needles, mp 131.5—132.0 °C, $[\alpha]_D^{25}$ −24.8° (c=2, CHCl$_3$). NMR (CDCl$_3$) δ: 2.68—2.96 (2H, m, $\mathrm{-CH\overset{O}{-}CH_2}$), 3.00—3.44 (3H, m, C$_4$-H, $\mathrm{-CH\overset{O}{-}CH_2}$), 3.92—4.52 (4H, m, C$_2$-H, -OCH$_2$CH), 5.28—5.52 (1H, m, C$_3$-H), 6.57—6.92 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1280 (ONO$_2$). *Anal.* Calcd for $C_{12}H_{13}NO_6$: C, 53.93; H, 4.90; N, 5.24. Found: C, 53.80; H, 4.87; N, 5.13.

(2'*R*),(3*R*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (*R*,*R*-NIP) (15)——Isopropylamine (40 ml, 470 mmol) and EtOH (80 ml) were mixed, and **18** (3.9 g, 14.6 mmol) was added. The mixture was stirred at a bath temperature of 70 °C for 1 h, then evaporated to dryness. The residue was dissolved in CHCl$_3$ (40 ml) and extracted with 1.8% aqueous AcOH (80 ml). The extract was washed with benzene (20 ml), made alkaline with 2 N NaOH and extracted with CHCl$_3$ (100 ml). The extract was washed with H$_2$O, and the solvent was evaporated off. The residue was purified by alumina column chromatography (solvent: CHCl$_3$), and then recrystallized from benzene-hexane to give 4.08 g (yield, 85.7%) of the desired product as colorless needles, mp 140.3—141.0 °C, $[\alpha]_D^{25}$ +15.5° (c=2, CHCl$_3$). NMR (CDCl$_3$) δ: 1.07 (6H, d, J=6 Hz, CH$_3$), 2.20—3.44 (7H, m, C$_4$-

H, $-CH_2NHCH$, OH), 3.92—4.16 (3H, m, $-OCH_2CH$), 4.16—4.54 (2H, m, C_2-H), 5.33—5.53 (1H, m, C_3-H), 6.58—6.92 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1278 (ONO_2). *Anal.* Calcd for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.79; N, 8.58. Found: C, 55.13; H, 6.81; N, 8.43.

(2′*S*),(3*R*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (*S*,*R*-NIP) (13) ——In the same manner as described for **15**, crude **13** was prepared from **17** (3.9 g, 14.6 mmol) and isopropylamine (40 ml, 470 mmol). Recrystallization from benzene–hexane gave 3.91 g (yield, 82.1%) of the pure product as colorless needles, mp 105.3—105.8 °C, $[\alpha]_D^{25}+16.2°$ ($c=2$, $CHCl_3$). NMR ($CDCl_3$) δ: 1.07 (6H, d, $J=6$ Hz, CH_3), 2.20—3.44 (7H, m, C_4-H, $-CH_2NHCH$, OH), 3.88—4.12 (3H, m, $-OCH_2CH$), 4.12—4.52 (2H, m, C_2-H), 5.32—5.52 (1H, m, C_3-H), 6.56—6.92 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1620, 1278 (ONO_2). *Anal.* Calcd for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.79; N, 8.58. Found: C, 55.19; H, 6.76; N, 8.53.

(2′*S*),(3*S*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (*S*,*S*-NIP) (14) ——In the same manner as described for **15**, crude **14** was prepared from **19** (3.9 g, 14.6 mmol) and isopropylamine (40 ml, 470 mmol). Recrystallization from AcOEt gave 3.51 g (yield, 73.6%) of the pure product as colorless needles, mp 140.5—141.0 °C, $[\alpha]_D^{25}-15.8°$ ($c=2$, $CHCl_3$). The NMR spectrum and IR spectrum of **14** were identical with those of **15**. *Anal.* Calcd for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.79; N, 8.58. Found: C, 55.32; H, 6.82; N, 8.55.

(2′*R*),(3*S*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (*R*,*S*-NIP) (16) ——In the same manner as describerd for **15**, crude **16** was prepared from **20** (3.9 g, 14.6 mmol) and isopropylamine (40 ml, 470 mmol). Recrystallization from benzene–hexane gave 3.93 g (yield, 82.6%) of the pure product as colorless needles, mp 105.3—106.0 °C, $[\alpha]_D^{25}-16.7°$ ($c=2$, $CHCl_3$). The NMR spectrum and IR spectrum of **16** were identical with those of **13**. *Anal.* Calcd for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.79; N, 8.58. Found: C, 55.27; H, 6.76; N, 8.70.

(2′*R*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (2′*R*-NIP) (10)—— Compound **1** (106 mg, 0.5 mmol) was dissolved in acetone (1 ml), and K_2CO_3 (69 mg, 0.5 mmol) and (*S*)-epichlorohydrin (93 mg, 1.0 mmol) were added. The mixture was stirred at 70 °C for 17 h. Purification of the reaction mixture by preparative thin layer chromatography (pTLC) gave **8** (31 mg) as colorless needles. This product (31 mg) was dissolved in EtOH (1.1 ml), and isopropylamine (1.1 ml, 12.9 mmol) was added. The mixture was stirred at 90 °C for 1 h, then purification by pTLC gave 34 mg (total yield, 20.1%) of **10** as a colorless viscous oil. $[\alpha]_D^{25}+2.6°$ ($c=1.5$, MeOH).

(2′*S*)-3,4-Dihydro-8-[2-hydroxy-3-(isopropylamino)propoxy]-3-nitroxy-2*H*-1-benzopyran (2′*S*-NIP) (9)——In the same manner as described for **10**, crude **9** was prepared from **1** (106 mg, 0.5 mmol), (*R*)-epichlorohydrin (93 mg, 1.0 mmol) and isopropylamine (1.1 ml, 12.9 mmol). Purification by pTLC gave 38 mg (total yield, 23.3%) of **9** as a colorless viscous oil. $[\alpha]_D^{25}$ 0° ($c=1.9$, MeOH).

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Studies on Positive Inotropic Agents. IV.[1] Synthesis of 5-(3-Amino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone Derivatives

MICHIAKI TOMINAGA, HIDENORI OGAWA,* EIYU YO, SHUJI YAMASHITA, YOUICHI YABUUCHI and KAZUYUKI NAKAGAWA

Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Kagasuno 463–10, Kawauchi-cho, Tokushima-shi 771–01, Japan

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Many 5-(3-amino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone derivatives were synthesized and examined for positive inotropic activity on the canine heart. These compounds were prepared by the reaction of 8-alkoxy-5-(2,3-epoxypropoxy)-2(1*H*)-quinolinone derivatives and various amines. Among them, 3,4-dihydro-8-hydroxy-5-[3-(1,1,3,3-tetramethylbutylamino)propoxy]-2(1*H*)-quinolinone hydrochloride (IIIf) was found to have potent positive inotropic activity with a relatively minor increase in heart rate.

Keywords——congestive heart failure; positive inotropic agent; 3,4-dihydro-8-hydroxy-5-[3-(1,1,3,3-tetramethylbutylamino)propoxy]-2(1*H*)-quinolinone; biological activity

We have been attempting to find positive inotropic agents among compounds bearing a 2(1*H*)-quinolinone nucleus as candidate drugs for the treatment of congestive heart failure. In the course of our studies, we found that various 2(1*H*)-quinolinone derivatives showed desirable activity.[1,2] We also found that 5-(3-*tert*-butylamino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone (8-OH-carteolol) increased cardiac contractile force.[3] Here we report the syntheses and biological activities on the canine heart of many 8-OH-carteolol derivatives.

Various 5-(3-amino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone derivatives (III) were synthesized from the epoxide (I)[4] by treatment with appropriate amines, followed by hydrolysis of the tetrahydropyranyloxy group (Table I). Treatment of I with methyl iodide in the presence of NaH in dimethyl formamide (DMF) gave 5-(2,3-epoxypropoxy)-3,4-dihydro-1-methyl-8-(2-tetrahydropyranyloxy)-2(1*H*)-quinolinone (IV). The 1-methyl derivative (V) was obtained from IV in the same manner as described for the synthesis of III.

The 8-benzyloxy derivative (II)[4] was converted to 8-benzyloxy-5-(2,3-epoxypropoxy)-2(1*H*)-quinolinone (VI) by dehydrogenation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in dioxane. Compound VI was reacted with butylamine in methanol, followed by hydrogenolysis of the benzyloxy group over 10% palladium on charcoal to afford the desired 2(1*H*)-quinolinone (VII) (Chart 1).

Treatment of 3,4-dihydro-5-hydroxy-8-methoxy-2(1*H*)-quinolinone (VIII)[5] with epichlorohydrin followed by reaction with benzylamine gave the quinolinone (XI). Reaction of the 5-acetoxy derivative (IX)[4] with 1-bromo-3-chloropropane in methanol in the presence of K_2CO_3 gave the corresponding chloride (X). 5-(3-Benzylaminopropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone hydrochloride (XII) was prepared by reaction of X with benzylamine in acetonitrile (Chart 2).

VI → 1) $BuNH_2$ 2) H_2-Pd/C → VII ·HCl

I: R^1=THP
II: R^1=CH_2Ph

I → DDQ → VI

I → 1) $HN(R^2)(R^3)$ 2) HCl → III ·HCl

I → MeI → IV

IV → 1) $BuNH_2$ 2) HCl → V ·HCl

Chart 1

VIII: R^1=Me, R^2=H
IX: R^1=THP, R^2=Ac

→ 1) CH_2-$CHCH_2Cl$ (epoxide) 2) $PhCH_2NH_2$ →

XI: R^1=Me, X=OH
XII: R^1=H, X=H

·HCl

IX → $Br(CH_2)_3Cl$, K_2CO_3 → X

X → 1) $PhCH_2NH_2$ 2) HCl → XII

Chart 2

TABLE I. 5-(3-Amino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone Derivatives (III)

Compd. No.	R^1	R^2	Yield[a] (%)	mp (°C) (Recrystn. solv.)	Formula	Analysis (%) Calcd (Found) C	H	N
IIIa	H	Et	27	227—229 (MeOH)	$C_{14}H_{20}N_2O_4 \cdot HCl$	53.08 (52.87	6.68 6.52	8.84 8.83)
IIIb	H	Pr	37	217—220 (MeOH)	$C_{15}H_{22}N_2O_4 \cdot HCl$	54.46 (54.39	7.01 6.74	8.47 8.43)
IIIc	H	Bu	26	214—217 (MeOH)	$C_{16}H_{24}N_2O_4 \cdot HCl$	55.73 (55.70	7.31 7.11	8.12 8.07)
IIId	H	Allyl	6.5	188—191 (EtOH)	$C_{15}H_{20}N_2O_4 \cdot HCl$	54.88 (54.78	6.44 6.25	8.52 8.67)
IIIe	H	$CH(Me)CH_2CH(Me)_2$	19	149—153 (iso-PrOH)	$C_{18}H_{28}N_2O_4 \cdot HCl \cdot 1/2H_2O$	56.61 (56.82	7.92 7.71	7.34 7.44)
IIIf	H	$C(Me)_2CH_2C(Me)_3$	7.6	217—219 (MeOH)	$C_{20}H_{32}N_2O_4 \cdot HCl$	59.91 (59.77	8.30 8.21	6.99 6.94)
IIIg	H	CH_2Ph	7.9	217—220 (iso-PrOH)	$C_{19}H_{22}N_2O_4 \cdot HCl$	60.02 (59.95	6.39 6.43	7.39 7.22)
IIIh	H	$(CH_2)_3Ph$	14	190—193 (MeOH)	$C_{21}H_{26}N_2O_4 \cdot HCl \cdot 1/2H_2O$	60.64 (60.46	6.79 6.74	6.74 6.68)
IIIi	H	C_6H_4-Me	25	206—209 (MeOH)	$C_{19}H_{22}N_2O_4 \cdot HCl$	60.24 (60.18	6.12 6.19	7.39 7.24)
IIIj	H	CH_2-C_6H_4-Me	7.1	219—222 (MeOH)	$C_{20}H_{24}N_2O_4 \cdot HCl$	61.14 (60.96	6.41 6.53	7.13 7.09)
IIIk	H	$C(Me)_2CH_2Ph$	9.2	170—173 (EtOH)	$C_{22}H_{28}N_2O_4 \cdot HCl \cdot H_2O$	60.20 (60.45	7.11 6.94	6.38 6.38)
IIIl	Me	CH_2Ph	40	194—196 (EtOH)	$C_{20}H_{24}N_2O_4 \cdot HCl$	61.14 (60.92	6.41 6.39	7.13 7.14)
IIIm		$-(CH_2)_5-$	34	265—267 (MeOH)	$C_{17}H_{24}N_2O_4 \cdot HCl \cdot 1/2H_2O$	55.81 (55.82	7.16 7.08	7.66 7.54)
IIIn		(piperazine ring) NCH_2Ph	31	246—249 (EtOH–H_2O)	$C_{23}H_{29}N_3O_4 \cdot 2HCl \cdot 1/2H_2O$	55.98 (56.02	6.54 6.31	8.52 8.60)

a) The yields from I (not optimized).

Biological Activity

The results of *in vitro* screening tests are shown in Table II. The inotropic and chronotropic effects of these compounds were compared with those of dobutamine.[6]

As regards effect on contractile force, the hydroxy group on the alkyl side chain is clearly important, since IIIg (having the hydroxy group) showed potent activity, while XII (without the hydroxy group) was inactive. Marked loss of activity was observed on alkylation of the phenolic hydroxy group or the amino group (see IIIl and XI). The influence of the

TABLE II. Biological Activities of 2(1*H*)-Quinolinone Derivatives on the Canine Heart ($n=1$)

Compd. No.	Inotropic effect	Chronotropic effect
IIIa	0.8	0.4
IIIb	0.1	LE
IIIc	0.2	0.1
IIId	3.3	2.6
IIIe	7.5	12
IIIf	6.5	0.5
IIIg	1.5	0.5
IIIh	2.1	0.6
IIIi	LE	LE
IIIj	0.9	0.3
IIIk	6.0	3.1
IIIl	LE	LE
IIIm	0.1	LE
IIIn	LE	LE
V	LE	LE
VII	8.0	4.2
XI	LE	LE
XII	LE	LE

The potency of inotropic and chronotropic effects of the test compounds was evaluated at doses (ED 50%) producing the half-maximal response to dobutamine as follows. Activity ratio of test compound = ED 50% of dobutamine/dose of test compound producing the same response as ED 50% of dobutamine. The larger the activity ratio; the more potent is the test compound. The highest dose (1 nmol) of dobutamine used in these experiments increased developed tension by about 50% of the basal tension, and increased sinus rate by about 15 beats/min. LE means lower than 0.1 activity ratio.

bulkiness of the *N*-substituent on the inotropic activity was seen with compounds IIIa—c, e—h and k; compounds bearing a bulky group (IIIe, f and k) were more potent than the others (IIIa—c and g—h).

Compounds IIIe, f, k and VII had over 5 times more potent inotropic activity than dobutamine. Among them, compound IIIf showed a slight positive chronotropic effect.

Experimental

All melting points are uncorrected. Infrared (IR) apectra were recorded on a JASCO IR-810 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390 instrument with tetramethylsilane or 3-(trimethylsilyl)propionic acid-d_5 as an internal standard.

Preparation of 5-(3-Alkylamino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone Derivatives (III). 5-(3-Allylamino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone Hydrochloride (IIId)——A mixture of 3,4-dihydro-5-(2,3-epoxypropoxy)-8-(2-tetrahydropyranyloxy)-2(1*H*)-quinolinone (3 g), allylamine (3.7 ml) and MeOH (20 ml) was stirred under reflux for 2 h, then concentrated *in vacuo*. The residue was dissolved in MeOH saturated with hydrogen chloride and concentrated *in vacuo*. The residue was recrystallized from EtOH to give IIId (0.21 g, 6.5%) as colorless needles, mp 188—191 °C. IR ν_{max}^{KBr} cm^{-1}: 1640, 1605, 1270. NMR (DMSO-d_6) δ: 2.25—3.52 (6H, m, $C\underline{H}_2C\underline{H}_2$, $C\underline{H}_2NH$), 3.62 (2H, d, $J=6$ Hz, $OC\underline{H}_2$), 3.71—4.45 (3H, m, $C\underline{H}$, $C\underline{H}_2CH=$), 5.22—5.65 (2H, m, $CH=C\underline{H}_2$), 5.74—6.12 (1H, m, $C\underline{H}=CH_2$), 5.85 (1H, d, $J=5$ Hz, OH), 6.61, 6.65 (each 1H, d, $J=8$ Hz, aromatic H), 8.76, 9.30 (each 1H, brs, OH, CONH), 9.07 (2H, brs, NH_2^+). The elemental analysis data are given in Table I.

Compounds IIIa—c, e—n were obtained in the same manner as described for IIId. The yield, melting points and elemental analysis data are listed in Table I.

5-(2,3-Epoxypropoxy)-3,4-dihydro-1-methyl-8-(2-tetrahydropyranyloxy)-2(1*H*)-quinolinone (IV)——A solution of I (3 g) in DMF (15 ml) was treated with NaH (0.57 g, 60% dispersion in oil) with stirring under a nitrogen atmosphere. The mixture was stirred at room temperature for 1 h, then a solution of methyl iodide (0.92 ml) in DMF (2 ml) was added dropwise. The mixture was stirred at room temperature for 2 h, poured into ice-water and extracted

with $CHCl_3$. The $CHCl_3$ solution was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$/MeOH (100:1) to give IV (1.1 g, 35%) as a yellow viscous oil. NMR ($CDCl_3$) δ: 1.31—2.25 (6H, m, $(C\underline{H}_2)_3$), 3.35 (3H, s, NCH_3), 2.27—4.41 (11H, m, $OC\underline{H}_2$, $C\underline{H}_2C\underline{H}_2$, $C\underline{H}_2C\underline{H}(O)C\underline{H}_2$), 5.31 (1H, t, J=4 Hz, CH), 6.50, 6.94 (each 1H, d, J=8 Hz, aromatic H). *Anal.* Calcd for $C_{18}H_{23}NO_4$: C, 68.12; H, 7.31; N, 4.41. Found: C, 67.85; H, 7.27; N, 4.51.

5-(3-Butylamino-2-hydroxypropoxy)-3,4-dihydro-8-hydroxy-1-methyl-2(1*H*)-quinolinone Hydrochloride (V)——A mixture of IV (4 g), butylamine (10 ml) and MeOH (20 ml) was stirred under reflux for 2 h. The reaction mixture was treated in the same manner as described for IIId to give V as colorless needles, mp 194—196 °C (EtOH). IR ν_{max}^{KBr} cm^{-1}: 1645, 1505, 1265. NMR (DMSO-d_6) δ: 0.90 (3H, t, J=7Hz, CH_3), 1.01—1.98 (4H, m, $C\underline{H}_2C\underline{H}_2$), 2.13—3.60 (8H, m, $C\underline{H}_2NHC\underline{H}_2$, $COC\underline{H}_2C\underline{H}_2$), 3.29 (3H, s, NCH_3), 3.90 (2H, d, J=5 Hz, $OC\underline{H}_2$), 3.95—4.50 (1H, m, CH), 5.83 (1H, d, J=5 Hz, OH), 6.63 (1H, d, J=8 Hz, aromatic H), 6.84 (1H, d, J=8 Hz, aromatic H), 8.96 (2H, br s, NH_2^+), 9.40 (1H, s, OH). *Anal.* Calcd for $C_{17}H_{26}N_2O_4 \cdot HCl$: C, 56.90; H, 7.58; N, 7.81. Found: C, 56.62; H, 7.40; N, 7.86.

8-Benzyloxy-5-(2,3-epoxypropoxy)-2(1*H*)-quinolinone (VI)——A mixture of 8-benzyloxy-5-(2,3-epoxypropoxy)-3,4-dihydro-2(1*H*)-quinolinone (II) (2.3 g), DDQ (1.9 g) and dioxane (25 ml) was stirred under reflux for 2 h. After removal of the solvent, the residue was dissolved in 1 N NaOH and $CHCl_3$. The $CHCl_3$ layer was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was recrystallized from MeOH to give VI (1.2 g, 52%) as a pale yellow powder, mp 138—140.5 °C. NMR (DMSO-d_6) δ: 2.62—3.07 (2H, m, $CHC\underline{H}_2O$), 3.16—3.65 (1H, m, $C\underline{H}CH_2O$), 3.79—4.53 (2H, m, $OC\underline{H}_2CH$), 5.02 (2H, s, $C\underline{H}_2$), 6.49 (1H, d, J=9 Hz, COCH), 6.66 (1H, d, J=8 Hz, aromatic H), 6.80—7.60 (6H, m, aromatic H), 8.08 (1H, d, J=9 Hz, $COCH=C\underline{H}$), 10.65 (1H, br s, CONH). *Anal.* Calcd for $C_{19}H_{17}NO_4$: C, 70.57; H, 5.30; N, 4.33. Found: C, 70.31; H, 5.18; N, 4.34.

5-(3-Butylamino-2-hydroxypropoxy)-8-hydroxy-2(1*H*)-quinolinone Hydrochloride (VII)——A mixture of VI (1.1 g) and butylamine (10 ml) in MeOH (20 ml) was stirred under reflux for 2 h, and concentrated. The residue was crystallized from Et_2O, then dissolved in MeOH saturated with hydrogen chloride. After removal of the solvent, the residue was mixed with 10% palladium on charcoal (0.1 g) and EtOH (20 ml). The mixture was stirred at room temperature under atmospheric pressure of hydrogen until the absorption of hydrogen ceased. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was recrystallized from MeOH to afford VII (0.34 g, 28%) as pale yellow needles, mp 217—220 °C. IR ν_{max}^{KBr} cm^{-1}: 1650, 1615, 1490, 1280. NMR (DMSO-d_6) δ: 0.91 (3H, t, J=7 Hz, CH_3), 1.02—1.93 (4H, m, $C\underline{H}_2C\underline{H}_2$), 2.73—3.60 (4H, m, $C\underline{H}_2NHC\underline{H}_2$), 3.99 (2H, d, J=5 Hz, $OC\underline{H}_2$), 4.03—4.50 (1H, m, CH), 5.90 (1H, d, J=5 Hz, OH), 6.44 (1H, d, J=9 Hz, COCH), 6.55 (1H, d, J=8 Hz, aromatic H), 6.94 (1H, d, J=8 Hz, aromatic H), 8.15 (1H, d, J=9 Hz, $COCH=C\underline{H}$), 9.01 (2H, br s, NH_2^+), 9.87, 10.50 (each 1H, br s, OH, CONH). *Anal.* Calcd for $C_{16}H_{22}N_2O_4 \cdot HCl \cdot 1/2H_2O$: C, 54.62; H, 6.88; N, 7.96. Found: C, 54.58; H, 6.79; N, 7.88.

5-(3-Benzylamino-2-hydroxypropoxy)-3,4-dihydro-8-methoxy-2(1*H*)-quinolinone Hydrochloride (XI)——A mixture of 3,4-dihydro-5-hydroxy-8-methoxy-2(1*H*)-quinolinone (VIII) (1.5 g), epichlorohydrin(4.3 g) and piperidine (0.1 g) was stirred at 95—100 °C for 2 h, and concentrated *in vacuo*. The residue was dissolved in dil. NaOH and $CHCl_3$. The $CHCl_3$ layer was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was mixed with benzylamine (2.2 g) and MeOH (10 ml), then stirred under reflux for 5 h. After removal of the solvent, the residue was chromatographed on silica gel with $CHCl_3$/MeOH (95:5), and the obtained fraction was dissolved in EtOH saturated with hydrogen chloride. The resulting precipitate was collected by filtration. Recrystallization from iso-PrOH gave XI (0.35 g, 11%) as colorless needles, mp 170—172 °C. IR ν_{max}^{KBr} cm^{-1}: 3230, 1680, 1505, 1260. NMR (DMSO-d_6) δ: 2.19—4.43 (11H, m, $COC\underline{H}_2C\underline{H}_2$, $OC\underline{H}_2C\underline{H}(OH)C\underline{H}_2$, $NHC\underline{H}_2$), 3.73 (3H, s, OCH_3), 5.90 (1H, br s, OH), 6.57 (1H, d, J=8 Hz, aromatic H), 6.82 (1H, J=8 Hz, aromatic H), 7.17—7.83 (5H, m, aromatic H), 8.91 (1H, s, CONH), 9.53 (2H, br s, NH_2^+). *Anal.* Calcd for $C_{20}H_{24}N_2O_4 \cdot HCl$: C, 61.14; H, 6.41; N, 7.13. Found: C, 60.93; H, 6.36; N 7.15.

5-(3-Chloropropoxy)-3,4-dihydro-8-(2-tetrahydropyranyloxy)-2(1*H*)-quinolinone (X)——A mixture of 5-acetoxy-3,4-dihydro-8-(2-tetrahydropyranyloxy)-2-(1*H*)-quinolinone (IX) (2 g), K_2CO_3 (2.6 g), 1-bromo-3-chloropropane (2 g) and MeOH (20 ml) was stirred under reflux for 3 h. The precipitates were removed by filtration and the filtrate was concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ and H_2O. The organic layer was washed with H_2O, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was recrystallized from iso-PrOH to afford X (1.3 g, 58%) as colorless prisms, mp 132—135 °C. NMR ($CDCl_3$) δ: 1.35—2.11 (6H, m, $-(C\underline{H}_2)_3$), 2.22—2.44 (2H, m, $C\underline{H}_2$), 2.51—3.13 (4H, m, $C\underline{H}_2C\underline{H}_2$), 3.60 (2H, t, J=6 Hz, $C\underline{H}_2Cl$), 3.74 (2H, t, J=6 Hz, $OC\underline{H}_2$), 3.76—4.20 (2H, m, $OC\underline{H}_2$), 5.25 (1H, t, J=4 Hz, CH), 6.69, 6.97 (each 1H, d, J=8 Hz, aromatic H), 8.05 (1H, br s, CONH). *Anal.* Calcd for $C_{17}H_{22}ClNO_4$: C, 60.08; H, 6.53; N, 4.12. Found: C, 59.88; H, 6.50; N, 3.96.

5-(3-Benzylaminopropoxy)-3,4-dihydro-8-hydroxy-2(1*H*)-quinolinone Hydrochloride (XII)——A mixture of X (4 g), NaI (2.6 g) and MeCN (40 ml) was heated under reflux for 2 h. Benzylamine (2.5 ml) and Et_3N (2 ml) were added to the mixture, and the whole was stirred under reflux for 4 h, then poured into 0.5 N NaOH solution (150 ml) and extracted with $CHCl_3$. The extract was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was treated with EtOH saturated with hydrogen chloride, and concentrated *in vacuo*. The residue was recrystallized from EtOH to give XII (0.8 g, 19%) as colorless needles, mp 251—254 °C. IR ν_{max}^{KBr} cm^{-1}: 3370, 1650, 1505, 1265. NMR (DMSO-d_6) δ: 1.96—3.77 (8H, m, $C\underline{H}_2C\underline{H}_2$, $NHC\underline{H}_2C\underline{H}_2$), 3.97 (2H, t, J=5 Hz, $OC\underline{H}_2$), 4.18 (2H, s, $NHC\underline{H}_2$),

6.44 (1H, d, $J=8$ Hz, aromatic H), 6.68 (1H, d, $J=8$ Hz, aromatic H), 7.29—7.80 (5H, m, aromatic H), 8.76, 9.34 (each 1H, br s, CONH, OH), 9.70 (2H, br s, NH_2^+). *Anal.* Calcd for $C_{19}H_{22}N_2O_3 \cdot HCl \cdot 1/2H_2O$: C, 61.37; H, 6.51; N, 7.53. Found: C, 61.28; H, 6.34; N, 7.58.

Pharmacological Evaluations——Inotropic and chronotropic effects of test compounds were examined by the use of isolated, blood-perfused dog heart preparations. The hearts were excised from mongrel dogs of either sex weighing 8—14 kg. The isolated, blood-perfused papillary muscle and sino-atrial node preparations were prepared according to the methods of Endoh and Hashimoto (1970)[7] and Kubota and Hashimoto (1973),[8] respectively. The preparations were cross-circulated through the cannulated arteries with blood from a donor dog anesthetized with sodium pentobarbital and receiving heparin. The perfusion pressure was kept constant at 100 mmHg. The papillary muscle was stimulated at a frequency of 2 Hz and tension developed by the muscle was measured with a force displacement transducer (Shinkoh, UL-20-240). Sinus rate was measured by the use of a cardiotachometer (Data Graph, T-149) triggered by developed tension of the right atrium. Blood flow through the cannulated arteries was measured with an electromagnetic flow meter (Nihon Kohden, MF-27). Recording of these parameters was done on an ink-writing rectigraph (Sanei Instrument, 8S). The compounds were injected intraarterially with microsyringes.

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Synthesis of 1,2,3,4-Tetrahydro-β-carboline Derivatives as Hepatoprotective Agents. IV. Positional Isomers of 1,2,3,4-Tetrahydro-2-methylthiothiocarbonyl-β-carboline-3-carboxylic Acid and Its 1-Alkylated Derivatives

YUTAKA SAIGA,[a] IKUO IIJIMA,*[,a] AKIHIKO ISHIDA,[a] TOSHIKAZU MIYAGISHIMA,[a] NORIO TAKAMURA,[a] TOKURO OH-ISHI,[a] MAMORU MATSUMOTO,[b] and YUZO MATSUOKA[b]

Organic Chemistry Research Laboratory[a] and Safety Research Laboratory,[b] Tanabe Seiyaku Co., Ltd., 2-2-50, Kawagishi, Toda-shi, Saitama 335, Japan

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Two tetrahydro-β-carboline-1- and -4-carboxylic acids (**1b, c**) and the corresponding hydroxymethyl derivatives (**2b, c**), which are positional isomers of the 3-carboxylic acid (**1a**) and its 3-hydroxymethyl derivative (**2a**), were synthesized and tested for hepatoprotective activity against carbon tetrachloride (CCl_4)-induced liver damage in mice. The hepatoprotective activity of these positional isomers decreased in the following order; **1a** > **1b** > **1c** > and **2a** > **2b** > **2c**.

The effect of alkyl substitution at the 1 position of **1a** and **2a** was also examined with the *cis* and *trans* isomers (**5a, b—14a, b**). Compounds with small alkyl groups such as Me and Et showed potent activity. Lengthening of the alkyl group generally caused a decrease in activity. In a series of the stereoisomers of the 3-carboxylic acids (**5a, b—9a, b**), the *cis* isomers tend to be more active than the *trans* counterparts.

Keywords——dithiocarbamate; tetrahydro-β-carboline; hepatoprotective activity; positional isomer; stereoisomer; structure-activity relationship

The synthesis and structure-activity relationships of a series of new 2-alkylthiothiocarbonyl-1,2,3,4-tetrahydro-β-carbolines as hepatoprotective agents were described in our previous papers.[1-3] As a result of an examination of the effects of substitution on the dithiocarbamate group,[2] C_3,[3] the benzene ring,[3] and the indole nitrogen,[3] the 3-carboxylic acid (**1a**) and the 3-hydroxymethyl derivative (**2a**) were found to exhibit the most potent hepatoprotective activity. In those studies, the presence of hydrophilic substituents such as carboxylic acid and hydroxymethyl groups at the 3 position was shown to be the most favorable.[3] Therefore, it became of interest to examine the effect of transposition of these functionalities to the 1 or 4 position of the β-carboline skeleton. This paper describes the synthesis and hepatoprotective activity of the 1- and 4-carboxylic acids (**1b, c**) and the corresponding hydroxymethyl derivatives (**2b, c**), which are positional isomers of **1a** and **2a**. The effect of alkyl substitution at the 1 position of **1a** and **2a** was also examined with the *cis* and *trans* isomers (**5a, b—14a, b**).

R, N, CS_2Me, N H

1a: $R=CO_2H$
2a: $R=CH_2OH$

Chart 1

3b: R=1-CO_2H
3c: R=4-CO_2Me
4b: R=1-CH_2OH
4c: R=4-CH_2OH

1b: R=1-CO_2H
1c: R=4-CO_2H
2b: R=1-CH_2OH
2c: R=4-CH_2OH

5a, b—9a, b
a=*cis*
b=*trans*

10a, b—14a, b
a=*cis*
b=*trans*

Chart 2

Chemistry

1,2,3,4-Tetrahydro-β-carboline-1-carboxylic acid (**3b**)[4] and the 1-hydroxymethyl derivative (**4b**)[5] were prepared according to the known procedure. The 4-methoxycarbonyl-β-carboline (**3c**) was obtained by Pictet–Spengler cyclization of the β-alanine derivative (**16**)[6] with formaldehyde. Lithium aluminum hydride ($LiAlH_4$) reduction of **3c** readily gave the 4-hydroxymethyl derivative (**4c**). The β-carbolines (**3b** and **4b, c**) were converted to the corresponding dithiocarbamates (**1b** and **2b, c**) by the reaction with carbon disulfide (CS_2) and methyl iodide (MeI) in the presence of sodium hydroxide (NaOH) or triethylamine (Et_3N). Similar treatment of the ester (**3c**) followed by alkaline hydrolysis gave the 4-carboxylic acid (**1c**).

16 3c 4c

17

		$R^2=CO_2H$	$R^2=CH_2OH$
18a, b:	R^1=Me	23a, b	28a, b
19a, b:	R^1=Et	24a, b	29a, b
20a, b:	R^1=Pr	25a, b	30a, b
21a, b:	R^1=Bu	26a, b	31a, b
22a, b:	R^1=CH_2OH	27a, b	32a, b

a=*cis*
b=*trans*

Chart 3

The *cis* and *trans* isomers of the 1-alkyl-β-carboline-3-carboxylic acid (**23**)[7] and the esters (**19**[8] and **22**[9]) were prepared by the reported procedure. The higher homologues (**20** and **21**) were similarly prepared by Pictet–Spengler cyclization of DL-tryptophan methyl ester (**17**) with butanal and pentanal, respectively. The resulting mixtures of the *cis* and *trans* isomers of the esters (**20a**, **b** and **21a**, **b**) were separated by column chromatography. Stereochemical assignment of these isomers was based on the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra. Cook *et al.*[10] reported that the C_1 and C_3 signals of the *trans*-1,3-disubstituted 1,2,3,4-tetrahydro-β-carbolines were clearly upfield from those of the corresponding *cis* isomers. The C_1 and C_3 signals of the *cis*-1-propyl derivative (**20a**) appeared at 52.54 and

Table I. Hepatoprotective Activities of the Positional Isomers (**1a, b, c** and **2a, b, c**)

Compd. No.	Hepatoprotective activity 100 mg/kg	10 mg/kg
1a[a]	AA	AA
1b	AA	D
1c	A	
2a[a]	AA	AA
2b	AA	C
2c	AA	D

a) The (*R*), (*S*), and (*RS*) isomers of these compounds proved to be equipotent. See reference 2.

Table II. Dithiocarbamate Derivatives of 1-Substituted Tetrahydro-β-carboline-3-carboxylic Acids

COOH
N-CSSMe
N H
R

Compd. No.	R	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	S	Hepatoprotective activity 100 mg/kg	10 mg/kg
5a	Me	134—137	50	$C_{15}H_{16}N_2O_2S_2\cdot$	48.98	4.38	7.37	16.87	AA	AA
		($CHCl_3$)		$1/2\,CHCl_3$	(48.74	4.26	7.32	17.01)		
5b	Me	173—176	71	$C_{15}H_{16}N_2O_2S_2\cdot$	55.95	5.58	8.16	18.67	AA	A
		($EtOH-H_2O$)		$1/2\,C_2H_5OH$	(55.91	5.66	8.16	18.84)		
6a	Et	198—200	53	$C_{16}H_{18}N_2O_2S_2$	57.46	5.42	8.38	19.17	AA	AA
		($EtOH-H_2O$)			(57.34	5.66	8.32	19.00)		
6b	Et	155—157	70	$C_{16}H_{18}N_2O_2S_2$	57.46	5.42	8.38	19.17	AA	D
		($EtOH-H_2O$)			(57.69	5.74	8.33	18.82)		
7a	Pr	Powder	59	$C_{17}H_{20}N_2O_2S_2$	58.59	5.78	8.04	18.40	AA	B
					(58.63	5.75	8.02	18.23)		
7b	Pr	Powder	41	$C_{17}H_{20}N_2O_2S_2\cdot$	58.19	6.24	7.54	17.26	AA	A
				$1/2\,C_2H_5OH$	(57.94	6.06	7.75	17.36)		
8a	Bu	100—105	71	$C_{18}H_{22}N_2O_2S_2$	59.64	6.12	7.73	17.69	A	
		($EtOH-H_2O$)			(59.43	6.11	7.54	17.42)		
8b	Bu	99—102	75	$C_{18}H_{22}N_2O_2S_2\cdot$	58.15	6.95	6.78	15.52	A	
		($EtOH-H_2O$)		$C_2H_5OH\cdot 1/4\,H_2O$	(58.09	6.70	6.88	15.24)		
9a	$-CH_2OH$	Powder	54	$C_{15}H_{16}N_2O_3S_2\cdot$	53.46	5.33	7.79	17.84	AA	AA
				$1/2\,C_2H_5OH$	(53.28	5.30	7.65	17.61)		
9b	$-CH_2OH$	187—188	34	$C_{15}H_{16}N_2O_3S_2$	53.55	4.79	8.33	19.06	A	
		($EtOH-H_2O$)			(53.68	4.80	8.25	19.00)		

56.54 ppm, respectively, while those of the *trans* counterpart (**20b**) appeared at 50.17 and 52.51 ppm. The corresponding 1-butyl derivatives (**21a, b**) behaved similarly (see Experimental). These upfield shifts in the *trans*-isomers are quite similar to those reported for the corresponding 1-ethyl-3-methoxycarbonyl derivatives (**19a, b**).[10] The esters (**20a, b** and **21a, b**) were hydrolyzed to the corresponding acids (**25a, b** and **26a, b**) without epimerization. The 3-hydroxymethyl derivatives (**28a, b—32a, b**) were obtained by sodium borohydride ($NaBH_4$) reduction[11] of **18a, b—22a, b** (Table IV). The β-carbolines (**23a, b—32a, b**) were converted to the *N*-methylthiothiocarbonyl derivatives (**5a, b—14a, b**) listed in Tables II and III in the usual manner.

Pharmacology and Structure–Activity Relationships

The dithiocarbamates prepared in the present study were tested for hepatoprotective activity against acutely CCl_4-induced liver damage in mice after oral administration by the method reported previously.[1] The results were evaluated according to the criteria defined previously[1] and are listed in Tables I—III.

With regard to the activity of the positional isomers (Table I), the hepatoprotective activity of the carboxylic acids decreased in the following order: **1a** > **1b** > **1c**. This is also the case for the corresponding hydroxymethyl derivatives (**2a—c**). This observation and the favorable effect of the presence of hydrophilic substituents at the 3 position appear to suggest that some neighboring group participation of the 3-substituent with the dithiocarbamate group is important for the manifestation of activity.

The effects of introduction of various alkyl groups at C_1 of **1a** and **2a** are summarized in

TABLE III. Dithiocarbamate Derivatives of 1-Substituted Tetrahydro-3-hydroxymethyl-β-carbolines

Compd. No.	R	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found)				Hepatoprotective activity	
					C	H	N	S	100 mg/kg	10 mg/kg
10a	Me	164—166 (EtOH–H_2O)	90	$C_{15}H_{18}N_2OS_2$	58.79 (58.69	5.92 5.80	9.14 9.19	20.93 20.94)	AA	AA
10b	Me	153—156 (EtOH–H_2O)	80	$C_{15}H_{18}N_2OS_2$	58.79 (58.67	5.92 5.97	9.14 9.20	20.93 21.03)	AA	A
11a	Et	177—180 (Et_2O)	65	$C_{16}H_{20}N_2OS_2$	59.96 (60.23	6.29 6.30	8.74 8.79	20.01 19.84)	AA	D
11b	Et	170—172 (EtOH–H_2O)	77	$C_{16}H_{20}N_2OS_2$	59.96 (60.00	6.29 6.29	8.74 8.63	20.01 19.78)	AA	D
12a	Pr	Powder	80	$C_{17}H_{22}N_2OS_2$	61.04 (60.83	6.63 6.55	8.37 8.32	19.17 19.31)	C	
12b	Pr	Powder	74	$C_{17}H_{22}N_2OS_2$	61.04 (60.87	6.63 6.78	8.37 8.33	19.17 19.20)	A	
13a	Bu	Powder	61	$C_{18}H_{24}N_2OS_2$	62.03 (62.15	6.94 6.87	8.04 7.92	18.40 18.16)	D	
13b	Bu	Powder	70	$C_{18}H_{24}N_2OS_2$	62.03 (62.28	6.94 6.90	8.04 7.87	18.40 18.21)	C	
14a	$-CH_2OH$	Powder	38	$C_{15}H_{18}N_2O_2S_2$ $1/2\,C_2H_5OH$	55.63 (55.45	6.13 6.01	8.11 8.20	18.56 18.78)	AA	B
14b	$-CH_2OH$	179—180 (EtOH–H_2O)	39	$C_{15}H_{18}N_2O_2S_2$	55.87 (55.81	5.63 5.64	8.69 8.53	19.89 19.87)	AA	B

TABLE IV. 1,3-Disubstituted 1,2,3,4-Tetrahydro-β-carbolines

Compd. No.	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
24a	236—237 (EtOH–H_2O)	72	$C_{14}H_{16}N_2O_2 \cdot C_2H_5OH$	66.19 (66.01	7.64 7.70	9.65 9.51)
24b	242—243 (EtOH–H_2O)	67	$C_{14}H_{16}N_2O_2 \cdot C_2H_5OH$	66.19 (66.12	7.64 7.50	9.65 9.58)
25a	225—226 (EtOH–H_2O)	81	$C_{15}H_{18}N_2O_2 \cdot H_2O$	65.20 (65.01	7.29 7.05	10.14 10.00)
25b	210—211 (EtOH–H_2O)	78	$C_{15}H_{18}N_2O_2 \cdot 1/2H_2O$	67.39 (67.53	7.16 6.98	10.48 10.45)
26a	215—216 (MeOH)	78	$C_{16}H_{20}N_2O_2 \cdot H_2O$	66.19 (66.12	7.63 7.62	9.65 9.62)
26b	204—205 (MeOH)	79	$C_{16}H_{20}N_2O_2 \cdot H_2O$	66.19 (66.40	7.63 7.39	9.65 9.88)
27a	241—242 (MeOH)	90	$C_{13}H_{14}N_2O_3 \cdot H_2O$	59.08 (59.34	6.10 6.01	10.60 10.39)
27b	231—233 (MeOH)	65	$C_{13}H_{14}N_2O_3 \cdot 1/2H_2O$	61.17 (61.01	5.92 5.81	10.97 10.82)
28a	176—178 (EtOH)	69	$C_{13}H_{16}N_2O$	72.19 (71.98	7.46 7.53	12.95 12.67)
28b	197—198 (MeOH)	81	$C_{13}H_{16}N_2O$	72.19 (72.10	7.46 7.50	12.95 12.79)
29a	205—206 (MeOH)	84	$C_{14}H_{18}N_2O$	73.01 (73.06	7.87 7.85	12.16 12.22)
29b	170.5—171 (MeOH)	85	$C_{14}H_{18}N_2O$	73.01 (73.17	7.87 7.88	12.16 12.20)
30a	218—219 (MeOH–H_2O)	75	$C_{15}H_{20}N_2O$	73.74 (73.86	8.25 8.18	11.46 11.52)
30b	170.5—171 (MeOH–H_2O)	64	$C_{15}H_{20}N_2O$	73.74 (73.62	8.25 8.26	11.46 11.48)
31a	222—223 (EtOH)	86	$C_{16}H_{22}N_2O$	74.38 (74.25	8.58 8.58	10.84 10.78)
31b	175—176 (EtOH)	80	$C_{16}H_{22}N_2O$	74.38 (74.55	8.58 8.48	10.84 10.89)
32a	Powder	63	$C_{13}H_{16}N_2O_2 \cdot H_2O$	62.38 (62.19	7.25 7.04	11.19 11.23)
32b	Powder	75	$C_{13}H_{16}N_2O_2 \cdot H_2O$	62.38 (62.15	7.25 7.18	11.19 11.12)

Tables II and III. As regards the size of alkyl groups, the compounds with small alkyl groups such as Me and Et showed potent activity. The activity of **5a**, **6a**, or **10a** is comparable to that of **1a** or **2a**. Lengthening of the alkyl group generally caused a decrease in activity. Introduction of a hydroxymethyl group at C_1 gave a favorable effect, as exemplified by the 1,3-*cis*-hydroxymethyl derivative (**9a**).

In a series of the stereoisomers of the 3-carboxylic acids (**5a**, **b**—**9a**, **b**), *cis* isomers tend to be more active than the *trans* counterparts (**5a** *vs*. **5b** and **6a** *vs*. **6b**). On the other hand, this tendency is not apparent in the stereoisomers of the 3-hydroxymethyl derivatives (**10a**, **b**—

14a, b).

After pharmacological evalution of the most potent compounds of this series,[1-3] the (S)-3-hydroxymethyl-2-methylthiothiocarbonyl derivative [(S)-**2a**] was selected for further study as a candidate for a clinically useful hepatoprotective agent. Compound (S)-**2a** exhibits potent hepatoprotective activity against liver damage acutely induced by galactosamine, bromobenzene, and α-naphthyl isothiocyanate in rats. It also exhibits a therapeutic effect on chronic liver damage induced by CCl_4 in rats. Further studies on (S)-**2a** as a possible hepatoprotective agent are in progress and will be reported in a separate paper.

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 on a JEOL PMS-60 instrument and ^{13}C-NMR spectra were recorded with a JEOL FX-100S spectrometer at 25 MHz. Chemical shifts are given as δ values from tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, t = triplet, and q = quartet. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument.

1,2,3,4-Tetrahydro-2-methylthiothiocarbonyl-β-carboline-1-carboxylic Acid (1b)——CS_2 (912 mg, 12 mmol) was added to a solution of **3b**[4] (2.16 g, 10 mmol) and 10 N NaOH (2 ml, 20 mmol) in dimethyl sulfoxide (DMSO) (6 ml), and the whole was stirred at room temperature for 10 min. After addition of MeI (1.7 g, 12 mmol), the mixture was stirred for 10 min, poured into water, and extracted with AcOEt. The aqueous layer was acidified with 10% HCl and extracted with AcOEt. The extract was dried over Na_2SO_4 and concentrated. The residue was recrystallized from aq. EtOH to give **1b** (1.24 g, 41%), mp 146—147 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3300, 1705. MS m/z: 306 (M^+), 258, 215. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 2.69 (3H, s). *Anal.* Calcd for $C_{14}H_{14}N_2O_2S_2$: C, 54.88; H, 4.61; N, 9.14; S, 20.93. Found: C, 54.73; H, 4.66; N, 9.08; S, 20.67.

1,2,3,4-Tetrahydro-2-methylthiothiocarbonyl-β-carboline-4-carboxylic Acid (1c)——A solution of **3c**·HCl (110 mg, 0.41 mmol), Et_3N (0.12 ml, 0.82 mmol), and CS_2 (0.03 ml, 0.5 mmol) in DMSO (1 ml) was stirred at room temperature for 1 h. MeI (0.03 ml, 0.5 mmol) was added, and the whole was stirred for 3 h. The mixture was diluted with water, extracted with AcOEt, and the extract was washed with water, dried over $MgSO_4$, and concentrated. The residue was dissolved in MeOH (3 ml), and 1 N NaOH (0.52 ml) was added. The mixture was stirred at room temperature overnight and evaporated. The residue was acidified with 10% HCl, extracted with AcOEt, and the extract was washed with water and dried over $MgSO_4$. The solvent was evaporated off to give an oil, which was crystallized from aq. EtOH to provide **1c** (101 mg, 80%), mp 202—204 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3330, 1700. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 2.68 (3H, s). MS m/z: 306 (M^+), 259, 215, 169. *Anal.* Calcd for $C_{14}H_{14}N_2O_2S_2$: C, 54.88; H, 4.61; N, 9.14; S, 20.93. Found: C, 54.97; H, 4.60; N, 9.06; S, 20.60.

The carboxylic acids (**5**—**9**) were synthesized in a manner similar to that described for **1b** and their physical properties are listed in Table II.

Methyl 1,2,3,4-Tetrahydro-1-hydroxymethyl-β-carboline-2-carbodithioate (2b)——A solution of **4b**[5] (380 mg, 1.9 mmol), CS_2 (0.13 ml, 2 mmol), and Et_3N (0.28 ml, 2 mmol) in MeOH (12 ml)–H_2O (4 ml) was stirred at room temperature for 30 min, and then MeI (0.1 ml, 2 mmol) was added. The mixture was stirred for 2.5 h, and the solvent was evaporated off under reduced pressure. The residue was dissolved in AcOEt, and this solution was washed with water, 5% HCl, and water, and then dried over Na_2SO_4. After removal of the solvent, the residue was triturated with aq. EtOH to give **2b** (400 mg, 73%) as a powder. ^{1}H-NMR ($CDCl_3$) δ: 2.68 (3H, s). MS m/z: 292 (M^+), 274, 261, 259, 244. *Anal.* Calcd for $C_{14}H_{16}N_2OS_2$: C, 57.50; H, 5.52; N, 9.58; S, 21.93. Found: C, 57.30; H, 5.67; N, 9.38; S, 21.67.

Methyl 1,2,3,4-Tetrahydro-4-hydroxymethyl-β-carboline-2-carbodithioate (2c)——This compound was prepared in 58% yield as a powder from **4c** under conditions analogous to those described above. ^{1}H-NMR ($CDCl_3$) δ: 2.69 (3H, s). MS m/z: 292 (M^+), 274, 259, 244. *Anal.* Calcd for $C_{14}H_{16}N_2OS_2 \cdot 1/3H_2O$: C, 56.36; H, 5.63; N, 9.39; S, 21.49. Found: C, 56.53; H, 5.38; N, 9.28; S, 21.56.

The hydroxymethyl compounds (**10**—**14**) were prepared in a manner similar to that described for **2b**. Their physical data are listed in Table III.

Methyl 1,2,3,4-Tetrahydro-β-carboline-4-carboxylate (3c) Hydrochloride——A solution of **16**[6] (2.04 g, 8 mmol) and 35% formalin (828 mg, 9.6 mmol) in MeOH (160 ml) was stirred at room temperature for 18 h. The mixture was diluted with ether, and the resulting crystals were collected by filtration and dried to give **3c** (2.04 g, 95%) as colorless needles, mp 223—225 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3160, 3100, 1730. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 3.73 (3H, s), 4.40 (2H, s). MS m/z: 230 (M^+), 215, 213, 201, 196. *Anal.* Calcd for $C_{13}H_{14}N_2O_2 \cdot HCl$: C, 58.54; H, 5.67; Cl, 13.29; N, 10.50. Found: C, 58.47; H, 5.48; Cl, 13.31; N, 10.46. The free base had mp 172—174 °C (AcOEt).

1,2,3,4-Tetrahydro-4-hydroxymethyl-β-carboline (4c) Oxalate——A solution of **3c** (1.115 g, 4.84 mmol) in tetrahydrofuran (THF) (34 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (365 mg) in THF, and the whole was stirred at room temperature for 2.5 h. An excess of the hydride was decomposed by addition of water, and the

insoluble material was filtered off. The filtrate was concentrated to give an oil, which was converted to the oxalate to give **4c**·oxalate (1.17 g, 83%), mp 227—228 °C (H_2O). IR ν_{max}^{Nujol} cm^{-1}: 3410, 3230, 1610. MS *m/z*: 202 (M^+), 184, 144. *Anal.* Calcd for $C_{12}H_{14}N_2O \cdot C_2H_2O_4$: C, 57.53; H, 5.52; N, 9.58. Found: C, 57.56; H, 5.40; N, 9.55.

Methyl *cis*-1,2,3,4-Tetrahydro-1-methyl-β-carboline-3-carboxylate (18a) Hydrochloride——$SOCl_2$ (71.6 ml, 0.99 mol) was added dropwise to a suspension of **23a**[7] (188 g, 0.82 mol) at −10 °C, and the mixture was refluxed for 4 h. After removal of the solvent, the residue was recrystallized from MeOH to give **18a**·HCl (218 g, 95%), mp 226—230 °C (dec.) (lit.[7] mp 228—230 °C).

Methyl *trans*-1,2,3,4-Tetrahydro-1-methyl-β-carboline-3-carboxylate (18b)——This compound was similarly prepared from **23b**[7] in 85% yield. mp 154—156 °C (MeOH–iso-Pr_2O). IR ν_{max}^{Nujol} cm^{-1}: 3340, 1730. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, d, J=6.7 Hz), 3.7 (3H, s). MS *m/z*: 244 (M^+), 229, 185, 169. *Anal.* Calcd for $C_{14}H_{16}N_2O_2$: C, 68.83; H, 6.60; N, 11.47. Found: C, 68.81; H, 6.61; N, 11.49.

Methyl *cis*-1,2,3,4-Tetrahydro-1-propyl-β-carboline-3-carboxylate (20a) and Methyl *trans*-1,2,3,4-Tetrahydro-1-propyl-β-carboline-3-carboxylate (20b)——A solution of **17** (33 g, 0.13 mol) and butanal (21.0 g, 0.29 mol) in MeOH (450 ml) was refluxed for 48 h and concentrated to one half of the initial volume. The resulting solid was collected by filtration to give **20a**·HCl (15.4 g, 38%, mp 205—207 °C from MeOH). The mother liquor was evaporated to dryness, and the residue was basified with 10% NH_4OH and extracted with $CHCl_3$. The extract was washed with water, dried over $MgSO_4$, and evaporated to give an oil, which was chromatographed on silica gel using $CHCl_3$ as the eluent. The first eluate gave an additional amount of **20a** (2.5 g, mp 98—100 °C from iso-PrOH–iso-Pr_2O. Total yield 45%). IR ν_{max}^{Nujol} cm^{-1}: 3420, 3200, 1740. ^{1}H-NMR ($CDCl_3$) δ: 3.77 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 52.54 (C_1), 56.54 (C_3). MS *m/z*: 272 (M^+), 229. *Anal.* Calcd for $C_{16}H_{20}N_2O_2$: C, 70.56; H, 7.40; N, 10.29. Found: C, 70.30; H, 7.36; N, 10.41. From the second eluate, **20b** (8.2 g, 23%, mp 116—118 °C from iso-Pr_2O) was obtained. IR ν_{max}^{Nujol} cm^{-1}: 3420, 1718. ^{1}H-NMR ($CDCl_3$) δ: 3.72 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 50.17 (C_1), 52.51 (C_3). MS *m/z*: 272 (M^+), 229. *Anal.* Calcd for $C_{16}H_{20}N_2O_2$: C, 70.56; H, 7.40; N, 10.29. Found: C, 70.72; H, 7.39; N, 10.25.

Methyl *cis*-1-Butyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (21a) and Methyl *trans*-1-Butyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylate (21b)——These compounds were prepared from **17** in a manner similar to that described above. The *cis* compound (**21a**); 53%, mp 85—87 °C (iso-Pr_2O–iso-PrOH). IR ν_{max}^{Nujol} cm^{-1}: 3340, 3240, 1740. ^{1}H-NMR ($CDCl_3$) δ: 3.80 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 52.80 (C_1), 56.60 (C_3). MS *m/z*: 286 (M^+). *Anal.* Calcd for $C_{17}H_{22}N_2O_2$: C, 71.30; H, 7.74; N, 9.78. Found: C, 71.26; H, 7.68; N, 9.75. The *trans* compound (**21b**); 25%, mp 102—103 °C (iso-Pr_2O–hexane). IR ν_{max}^{Nujol} cm^{-1}: 3320, 3150—3050, 1720. ^{1}H-NMR ($CDCl_3$) δ: 3.72 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 50.46 (C_1), 52.51 (C_3). MS *m/z*: 286 (M^+). *Anal.* Calcd for $C_{17}H_{22}N_2O_2$: C, 71.30; H, 7.74; N, 9.78. Found: C, 71.32; H, 7.73; N, 9.77.

***cis*-1-Ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic Acid (24a)**——A solution of **19a**[8] (5.0 g, 19.3 mmol) and 1 N NaOH (30 ml) in MeOH (30 ml) was stirred at room temperature for 1.5 h, and the solvent was evaporated off under reduced pressure. Then 5% HCl was added to the residue and the resulting solid was collected by filtration washed with water, and then dried. Recrystallization from aq. EtOH gave **24a** (3.4 g, 72%), mp 236—237 °C. IR ν_{max}^{Nujol} cm^{-1}: 3500, 3250, 1650. ^{1}H-NMR (DMSO-d_6–CF_3CO_2H) δ: 1.07 (3H, t, J=7.0 Hz), 3.47 (2H, q, J=7.0 Hz). MS *m/z*: 244 (M^+), 215, 169.

The carboxylic acids (**24b**, **25a**, **b**, **26a**, **b**, and **27a**, **b**) were similarly prepared and are listed in Table IV.

***cis*-1,2,3,4-Tetrahydro-3-hydroxymethyl-1-methyl-β-carboline (28a)**——$NaBH_4$ (7.0 g, 0.185 mol) was added to **18a**·HCl (13.2 g, 0.047 mol) in 85% aq. EtOH (150 ml) at 15—17 °C, and the mixture was stirred at room temperature for 10 h and then refluxed for 2 h. Insoluble material was filtered off, and the filtrate was concentrated. The residue was extracted with $CHCl_3$, and the extract was dried over Na_2SO_4. The solvent was evaporated off to give a solid, which was recrystallized from EtOH to afford **28a** (7.0 g, 69%), mp 176—178 °C. ^{1}H-NMR (DMSO-d_6) δ: 1.40 (3H, d, J=6.4 Hz). MS *m/z*: 216 (M^+), 201, 185, 183, 157.

The hydroxymethyl compounds (**28b**, **29a**, **b**, **30a**, **b**, **31a**, **b**, and **32a**, **b**) were prepared in a similar manner and are listed in Table IV.

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Studies on the Glycosides of *Epimedium grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. II[1]

TOSHIO MIYASE,*[a] AKIRA UENO,[a] NOBUO TAKIZAWA,[b]
HIROMI KOBAYASHI[b] and HIROKO OGUCHI[b]

Shizuoka College of Pharmacy,[a] 2-2-1, Oshika, Shizuoka 422, Japan and Central Research Laboratories, Yomeishu Seizo Co., Ltd.,[b] Nakaminowa, Minowa-cho, Kamiina-gun, Nagano 399-46, Japan

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Two new glycosides of ionone derivatives, icarisides B_3 (2) and B_4 (3), as well as a new phenylethanoid glycoside, icariside D_1 (4), and two new lignan glycosides, icarisides E_1 (12) and E_2 (13), have been isolated from *Epimedium grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI, together with eight known glycosides. The structures of the new compounds were established on the basis of chemical evidence and spectral data.

Keywords——*Epimedium grandiflorum* var. *thunbergianum*; ionone derivative; phenylethanoid glycoside; lignan glycoside; icariside B; icariside D; icariside E

In the previous paper,[1] we reported the isolation and the structure elucidation of ten glycosides from *Epimedium grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. Now we wish to report the structures of five new glycosides, icarisides B_3 (**2**), B_4 (**3**), D_1 (**4**), E_1 (**12**) and E_2 (**13**), which were isolated together with eight known glycosides, from the polar fraction of the water extract of *E. grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. The structures of these compounds were determined on the basis of chemical evidence and spectroscopic studies.

Compounds **1** (roseoside),[2] **5** ((+)-syringaresinol-*O*-β-D-glucopyranoside),[3] **6** (liriodendrin),[3,4] **7** ((−)-olivil-4''-*O*-β-D-glucopyranoside),[5] **8** (1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-hydroxyphenoxy]-1,3-propanediol),[6] **9** (1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-glucopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol),[7] **10** (1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol),[7,8] and **11** (1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol)[7,8] were identified by comparison of various data with reported values.

Icariside B_3 (**2**), $C_{19}H_{30}O_9$, $[\alpha]_D$ −95.8°, was obtained as colorless needles, mp 191—192 °C. The ultraviolet (UV), infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra were very similar to those of icariside B_2 (**2c**)[1] but in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **2**, downfield shifts were observed in comparison with the signals of icariside B_2 (**2c**) at C-1 (Δ+4.7 ppm), C-2 (Δ+30.2 ppm) and C-3 (Δ+4.0 ppm). Thus, C-2 carries an *O*-function. On acetylation, **2** afforded a pentaacetate **2b** whose ^{13}C-NMR spectrum showed a downfield shift of the C-2 signal (Δ+0.9 ppm) and upfield shifts of the C-1 (Δ−1.1 ppm) and C-3 (Δ−3.2 ppm) signals in comparison with those of **2**, suggesting that the glucosyl linkage is at the C-3 hydroxyl group in **2**. The circular dichroism (CD) spectrum of **2** showed a negative Cotton effect, $[\theta]_{232}$ −42800, suggesting a 5α,6α-epoxy structure.[1,9] On enzymatic hydrolysis, **2** afforded an aglycone **2a** whose ^{1}H-NMR spectrum showed two carbinol proton signals at δ 3.38 (br s, $W_{1/2}$=5.5 Hz) and 3.96

(m, $W_{1/2}=18.5$ Hz). The CD spectrum of **2a**, in the presence of a shift reagent, Eu(fod)$_3$, showed a positive Cotton effect, $[\theta]_{307}$ +47500 and a negative Cotton effect, $[\theta]_{285}$ −36000, suggesting that the absolute configurations at C-2 and C-3 are *S* and *R*, respectively.[10] These results led us to conclude the structure of icariside B_3 to be **2**.

Icariside B_4(**3**), $C_{19}H_{32}O_8 \cdot 1/2\, H_2O$, $[\alpha]_D$ −79.1°, was obtained as an amorphous powder. The ^{1}H-NMR spectrum showed three methyl signals at δ 1.17 (br s), 1.59 (br s) and 2.14 (s) and an anomeric proton signal at δ 4.80 (d, J=7 Hz). In the ^{13}C-NMR spectrum, nineteen carbon signals, including six signals due to a glucopyranosyl moiety were observed. Two oxygenated carbon signals due to an aglycone moiety were observed at δ 64.0 (d) and 76.3 (t), suggesting that the glucosyl linkage is at a primary hydroxyl group. On enzymatic hydrolysis, **3** afforded an aglycone **3a** whose ^{1}H-NMR spectrum showed the presence of a tertiary methyl (δ 1.02, br s), a vinyl methyl (δ 1.68, br s), an acetyl (δ 2.16, s), a hydroxy methyl (δ 3.36; 3.50, each d, J=11 Hz) and a secondary hydroxyl (δ 4.20, m, $W_{1/2}$=21 Hz) groups. The CD spectrum of the bis(*p*-dimethylaminobenzoate) of **3a** showed a negative Cotton effect, $[\theta]_{321}$ −67200 and a positive Cotton effect, $[\theta]_{295}$ +14700, suggesting that the absolute configurations of C-1 and C-3 are *S* and *R*, respectively, on the basis of the dibenzoate chirality rule and inspection of a Dreiding stereo model.[11] These results led us to conclude the structure of icariside B_4 to be **3**.

Icariside D_1 (**4**), $C_{19}H_{28}O_{10} \cdot H_2O$, $[\alpha]_D$ −89.6°, was obtained as a colorless syrup. The

1 : R=Glc
1a : R=Glc(OAc)$_4$

2 : R$_1$=OH, R$_2$=Glc
2a : R$_1$=OH, R$_2$=H
2b : R$_1$=OAc, R$_2$=Glc(OAc)$_4$
2c : R$_1$=H, R$_2$=Glc

3 : R=Glc
3a : R=H

4 : R=Glc$\overset{6}{—}$Api
4a : R=H

5 : R=H
6 : R=Glc

7

	R$_1$	R$_2$	R$_3$	R$_4$
8	Rham	H	H	H (*erythro*, *threo*)
8a	Rham(OAc)$_3$	Ac	H	Ac
9	Glc	Me	H	H (*erythro*)
9a	Glc(OAc)$_4$	Me	H	Ac
10	Rham	Me	H	H (*erythro*)
10a	Rham(OAc)$_3$	Me	H	Ac
11	Rham	Me	H	H (*threo*)
11a	Rham(OAc)$_3$	Me	H	Ac
11b	H	Me	H	H
12	Rham	Me	OH	H (*erythro*)
12a	Rham(OAc)$_3$	Me	OAc	Ac
13	Rham	Me	OH	H (*threo*)
13a	Rham(OAc)$_3$	Me	OAc	Ac

Chart 1

^{1}H-NMR spectrum showed a methylene signal at δ 3.00 (2H, t, J=7 Hz), two anomeric proton signals at δ 4.86 (1H, d, J=7 Hz) and 5.82 (1H, d, J=3 Hz) and an aromatic proton signal at δ 7.29 (5H, s). On acid hydrolysis, **4** afforded phenethyl alcohol as the aglycone (**4a**) and apiose and glucose as the sugar moiety. In the ^{13}C-NMR spectrum of **4**, the signal due to C-6 of glucose was shifted downfield by *ca.* 6 ppm and that of C-5 of glucose was shifted upfield by *ca.* 1 ppm in comparison with those of usual glucopyranosides.[12] Thus, apiose was

TABLE I. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	**2**[a]	**2a**[b]	**2b**[a]	**3**[a]	**3a**[b]
2		3.38 (1H, br s, $W_{1/2}$=5.5 Hz)			
3		3.96 (1H, m, $W_{1/2}$=18.5 Hz)			4.20 (1H, m, $W_{1/2}$=21 Hz)
4					
7	7.32 (1H, d, J=16 Hz)	7.03 (1H, d, J=16 Hz)	7.27 (1H, d, J=16 Hz)		
8	6.54 (1H, d, J=16 Hz)	6.31 (1H, d, J=16 Hz)	6.52 (1H, d, J=16 Hz)		
9					
10	2.31 (3H, s)	2.30 (3H, s)	2.33 (3H, s)	2.14 (3H, s)	2.16 (3H, s)
11	1.11 (3H, s)[c]	0.98 (3H, s)[d]	1.10 (3H, s)[e]	1.17 (3H, br s)	1.02 (3H, br s)
12	1.14 (3H, s)[c]	1.20 (3H, s)[d]	1.22 (3H, s)[e]		3.36 (1H, d, J=11 Hz) 3.50 (1H, d, J=11 Hz)
13	1.40 (3H, br s)	1.22 (3H, s)[d]	1.26 (3H, s)[e]	1.59 (3H, br s)	1.68 (3H, br s)
Anomeric	4.98 (1H, d, J=7 Hz)			4.80 (1H, d, J=8 Hz)	
OAc			2.02, 2.03, 2.09, 2.14, 2.17 (each 3H, s)		

Run at 89.55 MHz in *a*) pyridine-d_5, *b*) $CDCl_3$ solution. *c*—*e*) Assignments may be interchanged in each column.

TABLE II. ^{13}C-NMR Chemical Shifts

Carbon No.	**2**	**2b**	**3**	Carbon No.	**2**	**2b**	**3**
Aglycone moiety				Sugar moiety			
1	39.8	38.7	42.5	1′	104.0	99.4	105.2
2	75.0[a]	75.9	44.2[f]	2′	75.0	72.0[d]	75.1
3	75.5[a]	72.3[d]	64.0	3′	78.6[c]	73.4	78.7[h]
4	33.3	33.5	43.0[f]	4′	71.7	69.4	71.7
5	67.1	66.5	128.9[g]	5′	78.3[c]	72.0[d]	78.5[h]
6	69.1	69.4	133.7[g]	6′	62.7	62.5	62.8
7	144.0	142.0	22.4				
8	133.0	133.8	44.6[f]	OAc		20.5×2	
9	197.3	197.2	208.2			20.6×2	
10	27.6	27.7	29.8			20.9	
11	24.7[b]	23.3[e]	25.1			169.5, 169.9, 170.3,	
12	23.6[b]	23.1[e]	76.3			170.4, 170.6	
13	19.9	20.0	20.2				

Run at 22.5 MHz in pyridine-d_5 solution. *a*—*h*) Assignments may be interchanged in each column.

attached to C-6 of glucose. From these data, the structure of icariside D_1 was decided to be **4**.

Icariside E_1 (**12**), $C_{26}H_{36}O_{12} \cdot 1/2\ H_2O$, $[\alpha]_D$ $-24.3°$, was obtained as an amorphous powder. The UV spectrum showed absorption maxima at 279 (3.62) and 228 (sh 4.22) nm (log ε). The ^{13}C-NMR spectrum was similar to that of lignan glycoside **10**, but in the aromatic proton region of the ^{1}H-NMR spectrum of **12**, two *meta*-coupled doublet signals were observed at δ 6.54 and 6.87 (each 1H, br d, J=1.5 Hz). On acetylation, **12** afforded a heptaacetate **12a**. In the ^{1}H-NMR spectrum of **12a**, a benzylic methylene signal was long-range-coupled with two aromatic proton signals at δ 6.52 and 6.66 (each 1H, br d, J=1.5 Hz) on the basis of a decoupling experiment. A benzylic proton signal was observed at δ 6.04 (1H, d, J=4.1 Hz), indicating that **12** is a pure *erythro* isomer.[6,13] From the above data, the structure of icariside E_1 was decided to be **12**.

Icariside E_2 (**13**), $C_{26}H_{36}O_{12}$, $[\alpha]_D$ $-12.7°$, was obtained as an amorphous powder. The

TABLE III. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	**12**[a]	**12**[b]	**12**[c]
Aglycone moiety			
2	6.54 (1H, br d, J=1.5 Hz)	6.42 (br s)	6.66 (1H, br d, J=1.5 Hz)
6	6.87 (1H, br d, J=1.5 Hz)	6.42 (br s)	6.52 (1H, br d, J=1.5 Hz)
7	2.67 (2H, br t, J=7 Hz)	2.61 (2H, br t, J=7 Hz)	2.65 (2H, br dd, J=9; 7 Hz)
8	1.93 (2H, m)	1.88 (2H, m)	
2′,5′,6′	7.2—7.6 (3H, m)	6.7—7.1 (3H, m)	6.8—7.1 (3H, m)
7′			6.04 (1H, d, J=4.1 Hz)
8′		4.00 (1H, m)	
Sugar moiety			
1″		4.68 (1H, d, J=1 Hz)	4.73 (1H, br s)
4″			5.08 (1H, t, J=9 Hz)
6″	1.64 (3H, d, J=6 Hz)	1.25 (3H, d, J=6 Hz)	1.23 (3H, d, J=6 Hz)
OMe	3.72 (3H, s)	3.83 (3H, s)	3.82 (3H, s)
	3.75 (3H, s)	3.87 (3H, s)	3.87 (3H, s)
OAc			1.95, 2.02, 2.07, 2.11, 2.17, 2.18, 2.32 (each 3H, s)

Proton No.	**13**[a]	**13**[b]	**13a**[c]
Aglycone moiety			
2	6.55 (1H, br d, J=1.5 Hz)	6.43 (br s)	6.67 (1H, br d, J=1.5 Hz)
6	6.87 (1H, br d, J=1.5 Hz)	6.43 (br s)	6.53 (1H, br d, J=1.5 Hz)
7	2.70 (2H, br t, J=7 Hz)	2.62 (2H, br t, J=7 Hz)	2.65 (2H, br dd, J=9; 7 Hz)
8	1.97 (2H, m)	1.85 (2H, m)	
2′,5′,6′	7.2—7.6 (3H, m)	6.7—7.1 (3H, m)	7.02 (3H, br s)
7′			6.13 (1H, d, J=6.9 Hz)
8′			
Sugar moiety			
1″		4.68 (1H, d, J=6 Hz)	4.74 (1H, br s)
4″			5.08 (1H, t, J=9 Hz)
6″	1.64 (3H, d, J=6 Hz)	1.24 (3H, d, J=6 Hz)	1.21 (3H, d, J=6 Hz)
OMe	3.76 (3H, s)	3.86 (6H, s)	3.84 (6H, s)
	3.82 (3H, s)		
OAc			1.94, 2.00, 2.16, 2.20, 2.30 (each 3H, s)
			2.06 (6H, s)

Run at 89.55 MHz in *a*) pyridine-d_5, *b*) methanol-d_4, *c*) $CDCl_3$ solution.

TABLE IV. ^{13}C-NMR Chemical Shifts

Carbon No.	8[a]	9[a]	10[a]	11[a]	11a[a]	11b[b]	12[a]	13[a]
Aglycone moiety								
1	133.7	136.4	136.3	136.3	136.4	137.7	z)	135.4
2	117.5	113.9	113.8	113.6	113.7	113.7	110.7	110.0
3	147.9[c]	147.5[g]	147.5[i]	147.8[m]	z)	147.4[t]	152.6	152.5
4	z)	z)	z)	z)	151.3[q]	151.3	139.0	139.1
5	117.5	118.4	118.4	118.5	119.0	119.5	154.1	153.9
6	120.8	121.3	121.3	121.3	121.1	121.5	104.6	104.4
7	31.8[d]	32.2	31.9[j]	31.9[n]	31.3[r]	32.3	31.8[u]	31.8[w]
8	32.3[d]	32.2	32.4[j]	32.4[n]	32.1[r]	35.4	33.0[u]	33.1[w]
9	66.9	69.1	67.0	66.9	67.5	61.5	67.1	67.1
1′	138.4; 138.6	z)	z)	134.1	137.1	133.8	133.5	133.4
2′	111.7; 112.0	112.2	112.2	112.0	112.5	111.6	111.8	112.0
3′	146.5[c]	147.2[g]	147.3	147.6[m]	151.9[q]	148.0[t]	147.5	148.0
4′	z)	z)	z)	z)	146.8	146.7[t]	z)	z)
5′	116.3	116.1	116.1	116.1	123.6	115.2	116.3	116.4
6′	119.6	120.7	120.7	120.7	120.1	120.4	120.6	121.0
7′	73.6; 74.0[e]	73.7	73.8[k]	73.6[o]	75.2	73.6	73.9	74.1[x]
8′	89.7; 90.6	86.5	86.5	87.8	80.6	88.0	90.1	91.6
9′	61.5; 61.8	61.8	61.8	61.8	63.3	61.5	61.1	62.0
Sugar moiety								
1″	101.6	104.8	101.7	101.7	98.0		101.8	101.8
2″	72.9[f]	75.3	73.0[l]	72.9[p]	70.5[s]		73.0[v]	73.0[y]
3″	72.3[f]	78.7[h]	72.4[l]	72.4[p]	70.1[s]		72.4[v]	72.5[y]
4″	74.1[e]	71.8	74.1[k]	74.1[o]	71.5		74.1	74.3[x]
5″	69.8	78.5[h]	69.8	69.8	67.0		69.9	69.9
6″	18.6	63.0	18.7	18.7	17.8		18.7	18.7
OMe	55.9	56.0	56.0	55.9	56.0 × 2	56.2	56.1 × 2	56.2
		56.1	56.1	56.1		56.3		56.3
OAc					20.6			
					21.0 × 5			
					168.9, 170.0,			
					170.5, 170.3 × 3			

Run at 22.5 MHz in *a*) pyridine-d_5, *b*) acetone-d_6 solution. *c*—*y*) Assignments may be interchanged in each column. *z*) Overlapped with solvent signals.

spectral data were very similar to those of **12**, but in the ^{1}H-NMR spectrum of the heptaacetate **13a**, a benzylic proton signal was observed at δ 6.13 (1H, d, J=6.9 Hz), showing a downfield shift (by 0.09 ppm) and a large coupling constant compared with those of **12a**.[6,13] From these data, the structure of icariside E_2 was decided to be **13**, which is the *threo* isomer of **12**.

Olivil-type lignans have already been isolated from *Epimedium* species, but this is the first time that lignans of the other type have been isolated. The absolute configurations of lignan glycosides **8**—**13** remain to be investigated.

Experimental

Melting points were taken on a Yanaco MP-500 micromelting point apparatus and are uncorrected. Optical rotations were run on a JASCO DIP-140 digital polarimeter. IR spectra were run on a JASCO A-202 IR spectrometer and UV spectra on a Shimadzu UV-360 recording spectrometer. CD spectra were recorded on a JASCO J-20A spectropolarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FX-90Q NMR spectrometer (89.55 and 22.5 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Gas chromatography (GC) was done on a Shimadzu GC-4CM

gas chromatograph. High-performance liquid chromatography (HPLC) was done on a Kyowa Seimitsu model K880 instrument.

Isolation——Aerial parts of *E. grandiflorum* MORR. var. *thunbergianum* (MIQ.) NAKAI (15 kg), collected in summer 1985, in Niigata prefecture, Japan, were extracted twice with hot water. The extract was absorbed on Amberlite XAD-2 and the resin was eluted with methanol after being washed with water. After repeated chromatography of the methanol eluate (420 g) on silica gel with a chloroform–methanol system and HPLC on a Develosil ODS-10 column (20 × 250 mm) with a water–acetonitrile system, thirteen glycosides were isolated.

Roseoside (1)[2]——Amorphous powder (120 mg), $[\alpha]_D^{23}$ +80.4° (c=2.88, MeOH). *Anal.* Calcd for $C_{19}H_{30}O_8$: C, 59.05; H, 7.83. Found: C, 58.83; H, 7.73. UV λ_{max}^{MeOH} nm (log ε): 237 (4.02). CD (c=0.0072, MeOH): +56300 (240).

Icariside B$_3$ (2)——Colorless needles from methanol–ethyl acetate (100 mg), mp 191-192 °C, $[\alpha]_D^{25}$ −95.8° (c=2.50, MeOH). *Anal.* Calcd for $C_{19}H_{30}O_9$: C, 56.71; H, 7.51. Found: C, 56.53; H, 7.46. UV λ_{max}^{MeOH} nm (log ε): 232 (4.09). IR ν_{max}^{KBr} cm^{-1}: 3430, 1675, 1365, 1260, 1075. CD (c=0.0216, MeOH): −42800 (232). ^{1}H- and ^{13}C-NMR: Tables I and II.

Icariside B$_4$ (3)——Amorphous powder (250 mg), $[\alpha]_D^{25}$ −79.1° (c=0.79, MeOH). *Anal.* Calcd for $C_{19}H_{32}O_8 \cdot 1/2 H_2O$: C, 57.41; H, 8.37. Found: C, 57.63; H, 8.07. IR ν_{max}^{KBr} cm^{-1}: 3430, 1710, 1370, 1165, 1080, 1030. ^{1}H- and ^{13}C-NMR: Tables I and II.

Icariside D$_1$ (4)——Colorless syrup (80 mg), $[\alpha]_D^{25}$ −89.6° (c=0.72, MeOH). *Anal.* Calcd for $C_{19}H_{28}O_{10} \cdot H_2O$: C, 52.53; H, 6.96. Found: C, 52.25; H, 6.97. IR ν_{max}^{KBr} cm^{-1}: 3450, 1630, 1050. ^{1}H-NMR (pyridine-d_5) δ: 3.00 (2H, t, J=7 Hz, H$_2$-β), 4.86 (1H, d, J=7 Hz, H-1′), 5.82 (1H, d, J=3 Hz, H-1″), 7.29 (5H, s, aromatic H). ^{13}C-NMR (pyridine-d_5) δ: 36.7 (C-β), 65.7 (C-5″), 69.0 (C-6′), 70.7 (C-α), 71.8 (C-4′), 75.0 (C-2′, C-4″), 77.2 (C-5′), 77.9 (C-2″), 78.6 (C-3′), 80.5 (C-3″), 104.6 (C-1′), 111.2 (C-1″), 126.5 (C-4), 128.7; 129.5 (C-2, C-6/C-3, C-5), 139.5 (C-1).

(+)-Syringaresinol-*O*-β-D-glucopyranoside (5)[3]——Colorless needles from methanol (22 mg), mp 187—191 °C, $[\alpha]_D^{25}$ −24.0° (c=1.98, MeOH).

Liriodendrin (6)[4]——Colorless needles from pyridine–methanol (20 mg), mp 255—258 °C, $[\alpha]_D^{23}$ −48.5° (c=1.02, pyridine). UV λ_{max}^{MeOH} nm (log ε): 270 (3.14).

(−)-Olivil-4″-*O*-β-D-glucopyranoside (7)[5]——Amorphous powder (25 mg), $[\alpha]_D^{25}$ −66.4° (c=2.56, MeOH). *Anal.* Calcd for $C_{26}H_{34}O_{12} \cdot 1/2 H_2O$: C, 57.03; H, 6.44. Found: C, 56.91; H, 6.28.

1-(4-Hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-hydroxyphenoxy]-1,3-propanediol (8)[6]——Amorphous powder (115 mg), $[\alpha]_D^{23}$ −26.9° (c=0.26, MeOH). *Anal.* Calcd for $C_{25}H_{34}O_{11} \cdot H_2O$: C, 56.81; H, 6.87. Found: C, 56.58; H, 6.72. UV λ_{max}^{MeOH} nm (log ε): 228 (sh 4.15), 280 (3.80). ^{13}C-NMR: Table IV. This compound was a mixture of *erythro* and *threo* isomers in a ratio of 1 : 1.

1-(4-Hydroxy-3-methoxyphenyl)-2-[4-(3-glucopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol (*erythro*) (9)[7]——Amorphous powder (200 mg), $[\alpha]_D^{23}$ −19.6° (c=0.28, MeOH). *Anal.* Calcd for $C_{26}H_{34}O_{12} \cdot H_2O$: C, 56.11; H, 6.52. Found : C, 56.16; H, 6.52. UV λ_{max}^{MeOH} nm (log ε): 228 (4.12), 280 (3.69). IR ν_{max}^{KBr} cm^{-1}: 3420, 1605, 1515, 1265, 1070, 1020. ^{13}C-NMR: Table IV.

1-(4-Hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol (*erythro*) (10)[7,8]——Amorphous powder (250 mg), $[\alpha]_D^{25}$ −26.9° (c=0.26, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{11}$: C, 59,53; H, 6.92. Found: C, 59.59; H, 6.66. UV λ_{max}^{MeOH} nm (log ε): 228 (4.16), 280 (3.75). IR ν_{max}^{KBr} cm^{-1}: 3430, 1610, 1515, 1450, 1270, 1125, 1030. ^{13}C-NMR: Table IV.

1-(4-Hydroxy-3-methoxyphenyl)-2-[4-(3-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol (*threo*) (11)[7,8]——Amorphous powder (150 mg), $[\alpha]_D^{25}$ −29.5° (c=1.05, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{11} \cdot 1/2 H_2O$: C, 58.53; H, 6.99. Found: C, 58.33; H, 6.84. UV λ_{max}^{MeOH} nm (log ε): 230 (4.18), 280 (3.75). IR ν_{max}^{KBr} cm^{-1}: 3450, 1605, 1515, 1450, 1270, 1130, 1030. ^{13}C-NMR: Table IV.

Icariside E$_1$ (*erythro*) (12)——Amorphous powder (32 mg), $[\alpha]_D^{23}$ −24.3° (c=0.37, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{12} \cdot 1/2 H_2O$: C, 56.81; H, 6.79. Found: C, 56.84; H, 6.67. UV λ_{max}^{MeOH} nm (log ε): 228 (sh 4.22), 279 (3.62). IR ν_{max}^{KBr} cm^{-1}: 3400, 1590, 1515, 1460, 1350, 1270, 1230, 1090, 1040. ^{1}H- and ^{13}C-NMR: Tables III and IV.

Icariside E$_2$ (*threo*) (13)——Amorphous powder (38 mg), $[\alpha]_D^{23}$ −12.7° (c=0.55, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{12}$: C, 57.77; H, 6.71. Found: C, 57.89; H, 6.78. UV λ_{max}^{MeOH} nm (log ε): 228 (sh 4.16), 279 (3.61). IR ν_{max}^{KBr} cm^{-1}: 3400, 1590, 1520, 1460, 1360, 1275, 1235, 1040. ^{1}H- and ^{13}C-NMR: Tables III and IV.

Acetylation of Roseoside (1)——Roseoside (1) (12 mg) was dissolved in pyridine and acetic anhydride (each 0.3 ml), and the reaction mixture was left at room temperature overnight. After evaporation of the reagents *in vacuo*, the tetraacetate (**1a**) (12 mg) was obtained as a colorless syrup.

Enzymatic Hydrolysis of Icariside B$_3$ (2)——A solution of icariside B$_3$ (**2**) (25 mg) in water (1 ml) was treated with cellulase (20 mg) at 38 °C for 2 h. The reaction mixture was diluted with water and extracted with ethyl acetate 3 times. Ethyl acetate was evaporated off and the residue afforded an aglycone (**2a**) (4 mg) as a colorless syrup after purification by HPLC [YMC Pack A-212 C$_8$, H_2O–CH_3CN (85 : 15)]. MS m/z: 240 (M$^+$, trace), 165 (22), 150 (34), 125 (37), 124 (55), 123 (100), 109 (56). CD (c=0.024, CCl_4 with equimolar Eu(fod)$_3$): +47500 (307), −36000 (285). ^{1}H-NMR: Table I.

Acetylation of Icariside B$_3$ (2)——Icariside B$_3$ (**2**) (10 mg) was acetylated in the same manner as described for **1**. The pentaacetate (**2b**) (8 mg) was obtained as colorless needles, mp 163—164 °C, after recrystallization from

methanol. *Anal.* Calcd for $C_{29}H_{40}O_{14}$: C, 56.86; H, 6.58. Found: C, 56.87; H, 6.56. IR $\nu_{max}^{KBr}cm^{-1}$: 1760, 1750, 1675, 1370, 1250, 1220, 1085, 1040. ^{1}H- and ^{13}C-NMR: Tables I and II.

Enzymatic Hydrolysis of Icariside B_4 (3)——A solution of icariside B_4(3) (15 mg) was treated in the same manner as described for **2** to give the aglycone (**3a**) (3 mg) as a colorless syrup after purification by HPLC [Develosil ODS-10, H_2O–CH_3CN (73:27)]. $[\alpha]_D^{23}$ $-146.7°$ (c=0.15, MeOH). MS m/z: 226 (M^+, trace), 208 (M^+-H_2O, 8), 195 (32), 178 (41), 159 (53), 135 (23), 120 (71), 119 (100). ^{1}H-NMR: Table I. CD of bis(p-dimethylaminobenzoate). (c=0.021, MeOH): -67200 (321), $+14700$ (295).

Acid Hydrolysis of Icariside D_1 (4)——A solution of icariside D_1 (4) (0.5 mg) in 1% H_2SO_4 (0.1 ml) was heated in a boiling water bath for 5 min. The solution was passed through an Amberlite XAD-2 column. Apiose was detected in the water eluate by GC in the same manner as described in the previous paper[1] and phenethyl glucoside was detected in the methanol eluate by HPLC. Furthermore, a solution of the methanol eluate in 10% H_2SO_4 (0.1 ml) was heated in a boiling water bath for 10 min, then diluted with water and extracted with ethyl acetate. Glucose was detected in the water layer, and phenethyl alcohol in the ethyl acetate layer. GC conditions: column, 1.5% OV-17 (3 mm × 1 m); column temperature, 170 °C; carrier gas, N_2; t_R 9.5 min (apiitol acetate), column temperature, 210 °C; t_R 6.2 min (glucitol acetate). HPLC conditions: column, Develosil ODS-7 (4.6 mm × 25 cm); H_2O–CH_3CN (8:2) flow rate, 1.3 ml/min, t_R 5.2 min (phenethyl glucoside), H_2O–CH_3CN (75:25), 9.2 min (phenethyl alcohol).

Acetylation of the Lignans 8—13——Compounds **8** (2.5 mg), **9** (1.5 mg), **10** (2.5 mg), **11** (20 mg), **12** (3.5 mg) and **13** (5.5 mg) were acetylated in the same manner as described for **1** to give the acetates. **8a** (2.5 mg), colorless syrup. **9a** (1.5 mg), colorless syrup. **10a** (2.5 mg), colorless syrup. **11a** (22 mg), colorless syrup. ^{13}C-NMR: Table IV. **12a** (3.5 mg), colorless syrup. ^{1}H-NMR: Table III. **13a** (5.5 mg), colorless syrup. *Anal.* Calcd for $C_{40}H_{50}O_{19}$: C, 57.55; H, 6.04. Found: C, 57.36; H, 6.03. ^{1}H-NMR: Table III.

Enzymatic Hydrolysis of the Lignan 11——Compound **11** (19 mg) was hydrolyzed in the same manner as described for icariside B_3 (**2**). The aglycone (**11b**) (5 mg) was obtained as a colorless syrup after purification by HPLC [Develosil ODS-10, H_2O–CH_3CN (72:28)]. ^{1}H-NMR (acetone-d_6) δ: 1.80 (2H, m, H_2-8), 2.63 (2H, m, H_2-7), 3.57 (2H, t, J=6 Hz, H_2-9), 3.84; 3.89 (each 3H, s, OMe), 4.18 (1H, m, H-8′), 4.91 (1H, d, J=6 Hz, H-7′). ^{13}C-NMR: Table IV.

Acid Hydrolysis of the Glycosides 1—3, and 5—13——Each glycoside was hydrolyzed in the same manner as described in the previous paper.[1] Glucose was obtained from **1—3**, **5—7** and **9** and rhamnose from **8** and **10—13**; the sugars were detected as the alditol acetates by GC. Conditions: column, 1.5% OV-17 (3 mm × 1 m); carrier gas, N_2; column temperature, 210 °C; t_R 6.2 min (glucitol acetate), 180 °C; 8.2 min (rhamnitol acetate).

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Studies on the Constituents of *Scutellaria* Species. IX.[1] On the Flavonoid Constituents of the Root of *Scutellaria indica* L.[2]

YUKINORI MIYAICHI,[a] YOSHITAKA IMOTO,[a] TSUYOSHI TOMIMORI*,[a] and CHUN-CHING LIN[b]

School of Pharmacy, Hokuriku University,[a] 3 Ho, Kanagawa-machi, Kanazawa, 920–11, Japan and School of Pharmacy, Kaohsiung Medical College,[b] Kaohsiung, Taiwan, Republic of China

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Four new flavanones (I—IV) and a new flavone (V) were isolated from the root of *Scutellaria indica* L., together with 2(*S*)-5,2'-dihydroxy-7,8,6'-trimethoxyflavanone, (±)-5,2'-dihydroxy-6,7,6'-trimethoxyflavanone, 5,7-dihydroxy-8,2'-dimethoxy flavone, rivularin, 5,2',6'-trihydroxy-7,8-dimethoxyflavone, scutevurin, 5,7,4'-trihydroxy-8-methoxyflavone, wogonin, alpinetin, caldamomin and wogonin 7-*O*-glucuronide. Compounds I—V (Fig. 1) were identified based on spectral data and simple chemical modifications.

Keywords——*Scutellaria indica*; Labiatae; flavonoid; flavone; flavanone; chalcone; structure elucidation

Scutellaria indica L. is a perennial herb of the family Labiatae, which is widely distributed in Japan, Taiwan, China, Korea and Indo-China. The dried whole herb of this plant is a crude drug which is known as "han xin cao" (韓信草) in China and has been used as an antidotic, analgesic and hemostatic for the treatment of hemoptysis, hematemesis and other diseases.[3] So far, only a flavone glycoside, scutellarin, has been isolated as a constituent of this plant.[4] As a part of our studies on the flavonoid constituents of *Scutellaria* species, we have now examined this plant.

As described in the experimental part, four new flavanones (I—IV) and a new flavone (V) were isolated together with eleven known flavonoids (VI—XVI) from the ethanol extract of the root of this plant which was collected in Taiwan. This paper deals with their structural identification.

Compound I was obtained as colorless needles, mp 197 °C (dec.), $C_{16}H_{14}O_6$, Mg-HCl test (+). It gave the absorption bands of hydroxyl and conjugated carbonyl groups and benzene rings in the infrared (IR) spectrum. The ultraviolet (UV) spectrum of I was characteristic of the 5,7-dihydroxy flavanone series.[5] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of I showed the signals of one methoxyl (3.67 ppm), two hydroxyls (9.6 and 10.8 ppm), one chelated hydroxyl (11.93 ppm) and an ABX type grouping due to the C-2 (5.76 ppm) and C-3 protons (2.76 and 3.23 ppm). In the aromatic region of the spectrum, the signals of the remaining five protons occurred as a singlet (6.01 ppm, 1H) due to the A-ring proton, and a double doublet (7.23 ppm, 1H, $J=7.9$ and 7.5 Hz), a broad triplet (6.89 ppm, 1H, $J=7.5$ Hz) and two doublets (7.48 ppm, 1H, $J=7.5$ Hz; 6.92 ppm, 1H, $J=7.9$ Hz) due to the B-ring protons.

On methylation by Kuhn's method,[6] I gave a trimethyl ether, mp 174 °C (dec.), $C_{19}H_{20}O_6$, which was identical with 5,7,8,2'-tetramethoxyflavanone, prepared from 2(*S*)-5,7-dihydroxy-8,2'-dimethoxyflavanone.[7]

Compound I was, therefore, considered to be a monomethyl ether of 5,7,8,2′-tetrahydroxyflavanone. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of I, the methoxyl carbon signal appeared downfield at 60.4 ppm, which indicated the methoxyl to be on the C-8 carbon, being di-*ortho*-substituted by two oxygen functions.[8]

It is known that flavanones having 2(*S*)-configuration exhibit a positive Cotton effect due to n–π* transition (~330 nm) and a negative Cotton effect due to π–π* transition (270—290 nm) in the circular dichroism (CD) spectra.[9] The CD curve of I exhibited positive and negative maxima at 309 and 288 nm, respectively, which established the 2(*S*)-configuration. Based on the above findings, compound I was determined to be 2(*S*)-5,7,2′-trihydroxy-8-methoxyflavanone.

Compound II was obtained as colorless needles, mp 202 °C, $C_{15}H_{12}O_5$, Mg-HCl test (+). The IR spectrum gave absorption bands corresponding to hydroxyl and conjugated carbonyl groups and aromatic rings. The UV spectrum and diagnostic shifts suggested II to be a 5,7-dihydroxyflavanone derivative.[5] The ^{1}H-NMR spectrum showed the presence of one chelated hydroxyl (12.15 ppm), and C-2 (5.72 ppm) and C-3 (2.71, 3.23 ppm) protons. In the aromatic region of the spectrum, a pair of doublets (J=2.0 Hz) (5.94 ppm, 1H; 5.91 ppm, 1H) for the A-ring protons and two double doublets (7.21 ppm, 1H, J=7.6 and 7.8 Hz; 6.87 ppm, 1H, J=7.6 and 7.8 Hz) and two doublets (6.89 ppm, 1H, J=7.8 Hz; 7.44 ppm, 1H, J=7.8 Hz) due to the B-ring protons were seen. These chemical shifts and splitting patterns of the B-ring protons suggest that II possesses the same B-ring (2′-OH) as I. This was also supported by the ^{13}C-NMR spectrum, in which the carbon signals due to the B-ring of II were observed to be almost superimposable on those of I. The 2(*S*)-configuration was confirmed in the same way as in the case of I. From these results, the structure of II was determined to be 2(*S*)-5,7,2′-trihydroxyflavanone.

Compound III was obtained as colorless needles, mp 192 °C, $C_{17}H_{16}O_7$, Mg-HCl test (+), and gave the absorption bands of hydroxyl and conjugated carbonyl groups and benzene rings in the IR spectrum. The UV spectrum and diagnostic shifts suggested the presence of a hydroxyl group at the C-5 position and the absence of a free hydroxyl at the C-7 position.[5] The ^{1}H-NMR spectrum of III showed the signals of two methoxyls (3.66 and 3.87 ppm), two hydroxyls (8.87 and 9.11 ppm), one chelated hydroxyl (12.03 ppm) and an ABX type grouping due to the C-2 (5.70 ppm) and C-3 protons (2.80 and 3.18 ppm). The mass spectrum of III exhibited a fragment ion peak originating from the B-ring at m/z 136 ($C_{18}H_8O_2^+$). These findings indicated III to be a flavanone possessing one hydroxyl (on C-5) and two methoxyls in the A-ring and two hydroxyls in the B-ring.. In the aromatic region of the ^{1}H-NMR spectrum, the remaining four protons were observed as a singlet (6.25 ppm, 1H) a double doublet (6.62 ppm, 1H, J=2.0 and 9.3 Hz) and two doublets (6.75 ppm, 1H, J=9.3 Hz; 6.92 ppm, 1H, J=2.0 Hz). The former singlet could be assigned to the C-6 proton by long-range selective proton decoupling (LSPD)[10] in the ^{13}C-NMR spectrum as follows. In the ^{1}H non-decoupling ^{13}C-NMR spectrum of III, the signal of the carbon attached to an isolated aromatic hydrogen was observed at 93.0 ppm in the form of a double doublet (J=163.2 and 7.4 Hz), which changed to a doublet when the chelated hydroxyl proton at the C-5 position was selectively irradiated, indicating that the isolated aromatic proton was present at the position *ortho* (C-6) to the chelated hydroxyl (C-5). These data indicated that the substitution pattern of the A-ring was 5-hydroxy-7,8-dimethoxy. This was further confirmed by the ^{13}C-NMR spectrum of III, in which the signal pattern of the A-ring was almost identical with that of 5,2′-dihydroxy-7,8,6′-trimethoxyflavanone (VI).[7] The latter three signals were assigned to the C-3′, C-4′ and C-6′ protons, respectively, from their chemical shifts and coupling patterns. This was further confirmed by the ^{13}C–^{1}H shift correlation spectrum (COSY) and the ^{13}C–^{1}H long-range COSY.[11]

The 2(*S*)-configuration of III was confirmed in the same way as in the case of I.

Compound III was, therefore, determined to be 2(*S*)-5,2′,5′-trihydroxy-7,8-dimethoxyflavanone.

Compound IV was obtained as pale yellow needles, mp 143 °C (dec.), $C_{24}H_{26}O_{13}$, Mg-HCl test positive. It gave the absorption bands of hydroxyl and conjugated carbonyl groups and benzene rings in the IR spectrum. The UV spectrum and diagnostic shifts suggested the presence of a hydroxyl at the C-5 position and the absence of a free hydroxyl at the C-7 position.[5]

On methanolysis, IV yielded 5,2′-dihydroxy-7,8,6′-trimethoxyflavanone (VI),[7] 5,2′-dihydroxy-6,7,6′-trimethoxyflavanone (VII),[7] methyl glucuronopyranoside methylester and the methyl glycoside of glucurono-6,3-lactone.

The absence of a substituent at the C-6 position in IV was confirmed by the LSPD method.[10] The aglycone of IV is, therefore, VI, and VII was considered to be a secondary product formed from VI by ring isomerization (similar to the interconvertibility of hemiphloin and isohemiphloin by acid).[12]

In the ^{1}H- and ^{13}C-NMR spectra of IV, the anomeric proton signal at 5.00 ppm (d, J=6.3 Hz) and a set of carbon signals between 71.4 ppm and 170.3 ppm including an anomeric carbon signal at 101.1 ppm (d, J=164.7 Hz) indicated the presence of a β-glucuronopyranosyl unit.

The 2(*S*)-configuration of IV was confirmed in the same way as in the case of I. Thus, the structure of IV was established as 2(*S*)-5,2′-dihydroxy-7,8,6′-trimethoxyflavanone 2′-*O*-β-glucuronopyranoside.

Compound V was obtained as yellow needles, mp 247 °C (dec.), $C_{23}H_{22}O_{12}$, Mg-HCl test positive, and gave the absorption bands of hydroxyl and conjugated carbonyl groups and benzene rings in the IR spectrum. The UV spectrum and diagnostic shifts suggested the presence of a hydroxyl at the C-5 position and the absence of a free hydroxyl at the C-7 position.[5]

On methanolysis V yielded 5,7-dihydroxy-8,2′-dimethoxyflavone (VIII),[7] methyl glucuronopyranoside methylester and the methylglycoside of glucurono-6,3-lactone. In the ^{13}C-NMR spectrum of V, the signals due to the sugar moiety indicated the presence of a β-glucuronopyranoside unit in V.

Hence V was determined to be 5,7-dihydroxy-8,2′-dimethoxyflavone 7-*O*-β-glucuronopyranoside. This was confirmed by its ^{13}C-NMR spectrum, in which the signal patterns of the A-ring and the B-ring were almost identical with those of wogonin 7-*O*-β-glucuronopyranoside (XVI)[13] and 2′-*O*-methylskullcapflavone I,[7,14] respectively.

Compounds VI—XVI are known flavonoids and were identified as 2(*S*)-5,2′-dihydroxy-7,8,6′-trimethoxyflavanone,[7] (±)-5,2′-dihydroxy-6,7,6′-trimethoxyflavanone,[7] 5,7-dihydroxy-8,2′-dimethoxyflavone,[7] rivularin,[15] 5,2′,6′-trihydroxy-7,8-dimethoxyflavone,[16] scu-

	R_1	R_2	R_3	R_4	R_5
I:	H	OMe	H	H	H
II:	H	H	H	H	H
III:	Me	OMe	H	OH	H
IV:	Me	OMe	OMe	H	β-glc.UA.

V: R=β-glc.UA.

Fig. 1

tevurin,[16] 5,7,4′-trihydroxy-8-methoxyflavone,[17] wogonin,[7] alpinetin,[1] caldamomin[1] and wogonin 7-*O*-glucuronide,[7] respectively, by direct comparison with authentic samples.

Experimental

General Procedures——All melting points were determined on a Yanagimoto micro melting point apparatus and are recorded uncorrected. UV spectra were determined with addition of diagnostic reagents by standard procedures[5] on a Hitachi recording spectrophotometer, type 323. IR spectra in KBr disk were run on a JASCO IR-A-2 spectrometer. NMR spectra were taken in dimethyl sulfoxide-d_6 (DMSO-d_6) on a JEOL JNM-FX-100 spectrometer (^{1}H-NMR at 100 MHz and ^{13}C-NMR at 25 MHz), and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard (s, singlet; d, doublet; m, multiplet; br, broad). Electron impact-mass spectra (EI-MS) were taken on a JEOL JMS-DX-300 mass spectrometer. CD spectra were run on a JASCO J-20A automatic recording spectropolarimeter. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. Gas-liquid chromatography (GLC) was run on a Shimadzu GC-6AM unit with a flame ionization detector using a glass column (2 m × 4 mm i.d.) packed with 5% SE-30 on Chromosorb W (60—80 mesh); column temperature, programmed from 150 °C (20 min hold) to 240 °C at 5 °C/min. Thin layer chromatography (TLC) was carried out on Kieselgel 60 F 254 (Merck) with the following solvent systems: $CHCl_3$–MeOH–H_2O–AcOH (100:4:0.2:0.1) (TLC-1), *n*-hexane–acetone–AcOH (60:40:0.1) (TLC-2), $CHCl_3$–MeOH–H_2O–HCOOH (25:8:1:1) (TLC-3), AcOEt–methyl ethyl ketone–H_2O–HCOOH (18:9:1:1) (TLC-4). Spots were detected by spraying of dil. H_2SO_4 followed by heating.

Extraction and Separation——The dried root (330 g) of *Scutellaria indica* L., collected in Taiwan in 1985, was extracted with boiling EtOH. The EtOH extract was concentrated to dryness to give a residue (110.7 g), which was suspended in H_2O and successively extracted with Et_2O and *n*-BuOH. The Et_2O layer was concentrated and the residue (6.1 g) was chromatographed on silica gel (500 g) using *n*-hexane–acetone (10:1 → 1:1) as an eluent to give five fractions, fr. 1-5, in the order of elution. Fraction 1 gave VI. Fraction 2 was rechromatographed on silica gel (solvent: benzene) to give VII, VIII and XIII. Fraction 3 was subjected to rechromatography on silica gel [solvent: benzene–$CHCl_3$ (1:1)] to give I, II and IX. Fraction 4, containing a mixture of two flavonoids, was passed through a silica gel column [solvent: benzene–$CHCl_3$ (1:2)] to give III and XI. Fraction 5 was subjected to repeated chromatography on silica gel [solvent: benzene–AcOEt (10:1)] to give X, XII, XIV and XV. The *n*-BuOH-soluble portion was concentrated and the residue (18.3 g) was chromatographed on silica gel (500 g) with a gradient of $CHCl_3$–MeOH–H_2O–HCOOH (100:10:1:0.1 → 100:30:3:0.3) as an eluent to give IV, V and XVI. Yields: I (50 mg), II (30 mg), III (100 mg), IV (20 mg), V (20 mg), VI (100 mg), VII (15 mg), VIII (30 mg), IX (6 mg), X (4 mg), XI (6 mg), XII (5 mg), XIII (40 mg), XIV (10 mg), XV (5 mg), XVI (30 mg).

2(*S*)-5,7,2′-Trihydroxy-8-methoxyflavanone (I)——Colorless needles (MeOH/H_2O), mp 197 °C (dec.). *Anal.* Calcd for $C_{16}H_{14}O_6$: C, 63.57; H, 4.67. Found: C, 63.53; H, 4.69. Mg-HCl (+). *Rf*: 0.24 (TLC-1), 0.28 (TLC-2). UV λ_{max}^{MeOH} nm ($\log \varepsilon$): 240 sh (3.82), 291 (4.21), 342 (3.63); $\lambda_{max}^{MeOH-NaOMe}$ nm ($\log \varepsilon$): 241 sh (4.12), 329 (4.36); $\lambda_{max}^{MeOH-AlCl_3}$ nm ($\log \varepsilon$): 240 sh (3.91), 277 sh (3.56), 316 (4.34), 400 (3.60); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm ($\log \varepsilon$): 240 sh (3.91), 313 (4.31), 398 (3.58); $\lambda_{max}^{MeOH-NaOAc}$ nm ($\log \varepsilon$): 253 sh (3.74), 283 (3.59), 330 (4.35); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm ($\log \varepsilon$): 292 (4.10), 333 (4.01). IR ν_{max}^{KBr} cm^{-1}: 3488 (OH), 1641 (conjugated CO), 1614 (arom. C=C). ^{1}H-NMR: 3.67 (3H, s, OCH_3), 2.76 (1H, dd, J=17.0, 3.2 Hz, *cis* 3-H), 3.23 (1H, dd, J=17.0, 12.3 Hz, *trans* 3-H), 5.76 (1H, dd, J=12.3, 3.2 Hz, 2-H), 6.01 (1H, s, 6-H), 6.89 (1H, br t, J=7.5 Hz, 5′-H), 6.92 (1H, br d, J=7.9 Hz, 3′-H), 7.23 (1H, dd, J=7.9, 7.5 Hz, 4′-H), 7.48 (1H, d, J=7.5 Hz, 6′-H), *ca.* 9.6 (1H, br s, 2′-OH), *ca.* 10.8 (1H, br s, 7-OH), 11.93 (1H, s, 5-OH). ^{13}C-NMR: 74.3 (C-2), 41.1 (C-3), 196.4 (C-4), 158.6 (C-5), 95.9 (C-6), 159.9 (C-7), 128.5 (C-8), 154.6 (C-9), 101.8 (C-10), 124.9 (C-1′), 154.3 (C-2′), 115.6 (C-3′), 129.4 (C-4′), 119.1 (C-5′), 126.8 (C-6′), 60.4 (C-8-O$\underline{C}H_3$). MS *m/z* (%): 302 (M^+, 70), 269 ($C_{15}H_9O_5$, 100), 167 ($C_7H_3O_5$, 73). CD (c=0.005, MeOH) $[\theta]^{14}$ (nm): +16717 (309) (positive maximum), −59554 (288) (negative maximum).

Methylation of I by Kuhn's Method: CH_3I[6] (0.2 ml) and Ag_2O (50 mg) were added to a solution of I (8 mg) in *N*,*N*-dimethylformamide (DMF) (0.3 ml), and the reaction mixture was left for 20 h with occasional shaking. Then $CHCl_3$ was added, and after removal of the resulting precipitate by filtration, the filtrate was evaporated to dryness. The residue was chromatographed on silica gel (10 g) using benzene as an eluent to give crude Ia, which was recrystallized from MeOH to give Ia, colorless needles, mp 174 °C (dec.). This product was identical (UV, IR, ^{1}H- and ^{13}C-NMR, mixed fusion) with 5,7,8,2′-tetramethoxyflavanone.[7]

2(*S*)-5,7,2′-Trihydroxyflavanone (II)——Colorless needles (MeOH/H_2O), mp 202 °C. *Anal.* Calcd for $C_{15}H_{12}O_5$: C, 66.17; H, 4.44. Found: C, 66.32; H, 4.46. Mg-HCl (+). *Rf*: 0.19 (TLC-1), 0.29 (TLC-2). UV λ_{max}^{MeOH} nm ($\log \varepsilon$): 239 sh (3.83), 290 (4.23), 330 sh (3.56); $\lambda_{max}^{MeOH-NaOMe}$ nm ($\log \varepsilon$): 241 sh (4.11), 325 (4.40); $\lambda_{max}^{MeOH-AlCl_3}$ nm ($\log \varepsilon$): 239 sh (3.81), 275 sh (3.54), 313 (4.35), 380 (3.53); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm ($\log \varepsilon$): 239 sh (3.83); 275 sh (3.62), 311 (4.32), 379 (3.53); $\lambda_{max}^{MeOH-NaOAc}$ nm ($\log \varepsilon$): 253 (3.69), 278 sh (3.59), 326 (4.32); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm ($\log \varepsilon$): 291 (4.16), 323 sh (3.90). IR ν_{max}^{KBr} cm^{-1}: 3428 (OH), 1639 (conjugated CO), 1597 (arom. C=C). ^{1}H-NMR: 2.71 (1H, dd, J=17.1, 3.4 Hz, *cis* 3-H), 3.23 (1H, dd, J=17.1, 12.7 Hz, *trans* 3-H), 5.72 (1H, dd, J=12.7, 3.4 Hz, 2-H), 5.91 (1H, d, J=2.0 Hz, 6-H), 5.94 (1H, d, J=2.0 Hz, 8-H), 6.87 (1H, dd, J=7.8, 7.6 Hz, 5′-H), 6.89 (1H, br d, J=7.8 Hz, 3′-H), 7.21 (1H, dd, J=7.8,

7.6 Hz, 4′-H), 7.44 (1H, d, J=7.8 Hz, 6′-H), 12.15 (1H, s, 5-OH), ^{13}C-NMR: 74.0 (C-2), 41.1 (C-3), 196.5 (C-4), 163.7 (C-5), 95.9 (C-6), 166.8 (C-7), 95.1 (C-8), 163.4 (C-9), 101.8 (C-10), 124.9 (C-1′), 154.4 (C-2′), 115.6 (C-3′), 129.5 (C-4′), 119.2 (C-5′), 127.1 (C-6′). MS m/z (%): 272 (M^+, 60), 254 ($C_{15}H_{10}O_4$, 98), 153 ($C_6H_1O_5$, 100). CD (c=0.005, MeOH) $[\theta]^{14}$ (nm): +9301 (309) (positive maximum), −62006 (283) (negative maximum).

2(*S*)-5,2′,5′-Trihydroxy-7,8-dimethoxyflavanone (III)——Colorless needles (MeOH/H_2O), mp 192 °C. *Anal.* Calcd for $C_{17}H_{16}O_7$: C, 61.44; H, 4.85. Found: C, 61.49; H, 4.87. Mg-HCl (+). *Rf*: 0.15 (TLC-1), 0.21 (TLC-2). UV λ_{max}^{MeOH} nm (log ε): 240 sh (3.89), 292 (4.13); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 243 (4.11), 288 (4.00), 315 sh (3.76), 390 (3.75); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 314 (4.22), 404 (3.56); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 310 (4.21), 396 (3.49); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 292 (4.10), 345 (3.48); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 292 (4.11), 345 (3.50). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1639 (conjugated CO), 1580 (arom. C=C). ^{1}H-NMR: 3.66, 3.87 (each 3H, each s, OCH_3 × 2), 2.80 (1H, dd, J=19.0, 3.9 Hz, *cis* 3-H), 3.18 (1H, dd, J=19.0, 12.9 Hz, *trans* 3-H), 5.70 (1H, dd, J=12.9, 3.9 Hz, 2-H), 6.25 (1H, s, 6-H), 6.62 (1H, dd, J=9.3, 2.0 Hz, 4′-H), 6.75 (1H, d, J=9.3 Hz, 3′-H), 6.92 (1H, d, J=2.0 Hz, 6′-H), 8.87, 9.11 (each 1H, each s, 5′ and 2′-OH), 12.03 (1H, s, 5-OH). ^{13}C-NMR: 74.5 (C-2), 41.5 (C-3), 197.1 (C-4), 159.0 (C-5), 93.0 (C-6, $J_{(C\text{-}6)-(6\text{-}H)}$=163.2 Hz, $J_{(C\text{-}6)-(5\text{-}OH)}$=7.4 Hz), 161.1 (C-7), 129.3 (C-8), 153.9 (C-9), 102.4 (C-10), 125.5 (C-1′), 146.5 (C-2′), 116.4 (C-3′), 115.9 (C-4′), 150.1 (C-5′), 113.2 (C-6′), 56.3 (C-7-O$\underline{C}H_3$), 60.5 (C-8-O$\underline{C}H_3$). MS m/z (%): 332 (M^+, 58), 299 ($C_{16}H_{11}O_6$, 100), 136 ($C_8H_8O_2^+$, 25) CD (c=0.005, MeOH) $[\theta]^{14}$ -nm): +9501 (308) (positive maximum), −54292 (285) (negative maximum).

2(*S*)-5,2′-Dihydroxy-7,8,6′-trimethoxyflavanone 2′-*O*-β-Glucuronopyranoside (IV)——Pale yellow needles (MeOH/H_2O) mp 143 °C (dec.). $[\alpha]_D^{14}$ −71.8° (c=0.03, MeOH). *Anal.* Calcd for $C_{24}H_{26}O_{13}$: C, 55.18; H, 5.03. Found: C, 55.05; H, 5.06. Mg-HCl (+). *Rf*: 0.41 (TLC-3), 0.32 (TLC-4). UV λ_{max}^{MeOH} nm (log ε): 241 sh (3.97), 289 (4.19), 347 (3.55); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 250 sh (4.14), 287 (4.18), 370 (3.84); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 280 sh (3.72), 314 (4.30), 404 (3.58); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 280 sh (3.74), 312 (4.29), 400 (3.60); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 289 (4.17), 349 (3.53); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 289 (4.17), 346 (3.54). IR ν_{max}^{KBr} cm^{-1}: 3452 (OH), 1740 (COOH), 1637 (conjugated CO), 1603 (arom. C=C). ^{1}H-NMR: 3.55, 3.80, 3.85 (each 3H, each s, OCH_3 × 3), 2.44 (1H, dd, J=13.7, 2.7 Hz, *cis* 3-H), 3.8—4.2 (m, *trans* 3-H), 5.00 (1H, d, J=6.3 Hz, anomeric H of glucuronic acid unit), 6.07 (1H, dd, J=13.9, 2.7 Hz, 2-H), 6.22 (1H, s, 6-H), 6.82 (2H, d, J=8.3 Hz, 3′,5′-H), 7.38 (1H, t, J=8.3 Hz, 4′-H), 12.16 (1H, s, 5-OH). ^{13}C-NMR: 71.5 (C-2), 39.4 (C-3), 198.2 (C-4), 159.3 (C-5), 92.6 (C-6, $J_{(C\text{-}6)-(6\text{-}H)}$=162.5 Hz, $J_{(C\text{-}6)-(5\text{-}OH)}$=6.6 Hz), 161.1 (C-7), 129.2 (C-8), 154.8 (C-9), 102.5 (C-10), 114.8 (C-1′), 156.8 (C-2′), 108.6 (C-3′), 131.0 (C-4′), 106.4 (C-5′), 159.0 (C-6′), 101.1 (C-1″, J=164.7 Hz), 73.2 (C-2″), 75.7 (C-3″), 71.4 (C-4″), 75.9 (C-5″), 170.3 (C-6″), 56.2 (C-6′-O$\underline{C}H_3$), 56.4 (C-7-O$\underline{C}H_3$), 60.6 (C-8-O$\underline{C}H_3$). MS m/z (%): 346 ($C_{18}H_{18}O_7$, 67), 328 ($C_{18}H_{16}O_6$, 50), 313 ($C_{17}H_{13}O_6$, 100). CD (c=0.005, MeOH) $[\theta]^{14}$ (nm): +6812 (309) (positive maximum), −34058 (285) (negative maximum).

Methanolysis of IV: A solution of IV (10 mg) in 10% HCl–MeOH (2 ml) was heated under reflux on a water bath for 3 h. The reaction mixture was neutralized with Ag_2CO_3. The precipitates were filtered off and the filtrate was concentrated to give the residue. The residue was crystallized from MeOH/H_2O to give a mixture of two types of crystals, which was chromatographed on silica gel (10 mg) using benzene as an eluent to give pale yellow needles (MeOH), mp 202 °C (dec.) and colorless needles (MeOH), mp 221 °C (dec.). They were identified as compounds VI and VII, respectively, by direct comparisons (TLC, UV, IR, ^{1}H- and ^{13}C-NMR, mixed fusion), with authentic specimens. The mother liquor of crystallization was shown to contain methyl glucuronopyranoside methyl ester [t_R 13 min 24 s (both α and β)] and the methyl glycoside of glucurono-6,3-lactone [t_R 6 min 05 s (α, trace), 6 min 48 s (β)] by GLC (as the trimethylsilylether derivatives).

5,7-Dihydroxy-8,2′-dimethoxyflavone 7-*O*-β-Glucuronopyranoside (V)——Yellow needles (MeOH) mp 247 °C (dec.). $[\alpha]_D^{14}$: −45.9° (c=0.04, MeOH). *Anal.* Calcd for $C_{23}H_{22}O_{12}$: C, 58.23; H, 4.68. Found: C, 58.29; H, 4.66. Mg-HCl (+). *Rf*: 0.35 (TLC-3), 0.26 (TLC-4). UV λ_{max}^{MeOH} nm (log ε): 275 (4.41), 333 (4.04); $\lambda_{max}^{MeOH-NaOMe}$ nm (log ε): 276 (4.39), 283 sh (4.37), 320 sh (4.33), 380 (3.74); $\lambda_{max}^{MeOH-AlCl_3}$ nm (log ε): 255 (3.94), 286 (4.37), 295 sh (4.34), 345 (4.07), 404 (3.84); $\lambda_{max}^{MeOH-AlCl_3-HCl}$ nm (log ε): 284 (4.36), 295 sh (4.31), 342 (4.07), 405 (3.78); $\lambda_{max}^{MeOH-NaOAc}$ nm (log ε): 275 (4.40), 333 (4.03); $\lambda_{max}^{MeOH-NaOAc-H_3BO_3}$ nm (log ε): 275 (4.40), 333 (4.03). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1714 (COOH), 1653 (conjugated CO), 1607, 1565 (arom. C=C). ^{1}H-NMR: 3.86, 3.91 (each 3H, each s, OCH_3 × 2), 5.29 (1H, br s, anomeric H of glucuronic acid unit), 6.90 (1H, s, 3-H), 6.70 (1H, s, 6-H), 7.15 (1H, br t, J=7.6 Hz, 5′-H), 7.22 (1H, br d, J=7.9 Hz, 3′-H), 7.57 (1H, br t, J=7.9 Hz, 4′-H), 7.86 (1H, dd, J=7.6, 1.2 Hz, 6′-H), 12.54 (1H, s, 5-OH). ^{13}C-NMR: 161.9 (C-2), 109.8 (C-3), 182.5 (C-4), 156.2 (C-5), 98.7 (C-6, $J_{(C\text{-}6)-(6\text{-}H)}$=163.3 Hz, $J_{(C\text{-}6)-(5\text{-}OH)}$=7.4 Hz), 156.2 (C-7), 129.5 (C-8), 149.5 (C-9), 105.4 (C-10), 119.5 (C-1′), 158.1 (C-2′), 112.8 (C-3′), 133.5 (C-4′), 121.1 (C-5′), 129.1 (C-6′), 99.9 (C-1″, J=163.3 Hz), 73.1 (C-2″), 75.5 (C-3″), 71.4 (C-4″), 75.9 (C-5″), 170.2 (C-6″), 56.0 (C-2′-O$\underline{C}H_3$), 61.5 (C-8-O$\underline{C}H_3$). MS m/z (%): 314 ($C_{17}H_{14}O_6$, 100), 299 ($C_{16}H_{11}O_6$, 70).

Methanolysis of V: V (10 mg) was methanolyzed and worked up in the same way as described for IV to give compound VIII and a sugar fraction, which was shown to contain methyl glucuronopyranoside methyl ester and the methyl glycoside of glucurono-6,3-lactone by GLC.

Identification of VI—XVI——VI (mp 202 °C (dec.)), VII (mp 221 °C (dec.)), VIII (mp 231 °C), IX (mp 259 °C), X (mp 286 °C (dec.)), XI (mp 278 °C (dec.)), XII (mp 302 °C), XIII (mp 203 °C), XIV (mp 222 °C), XV (mp 198 °C), XVI (mp 270 °C (dec.)) were identified as 2(*S*)-5,2′-dihydroxy-7,8,6′-trimethoxyflavanone, (±)-5,2′-dihydroxy-6,7,6′-

trimethoxyflavanone, 5,7-dihydroxy-8,2′-dimethoxyflavone, rivularin, 5,2′,6′-trihydroxy-7,8-dimethoxyflavone, scutevurin, 5,7,4′-trihydroxy-8-methoxyflavone, wogonin, alpinetin, caldamomin and wogonin 7-*O*-glucuronide, respectively, by direct comparisons with authentic specimens (UV, IR, ^{1}H- and ^{13}C-NMR, mixed fusion).

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Metabolism of Paeoniflorin and Related Compounds by Human Intestinal Bacteria. II.[1] Structures of 7*S*- and 7*R*-Paeonimetabolines I and II Formed by *Bacteroides fragilis* and *Lactobacillus brevis*

YUE-ZHONG SHU,[a] MASAO HATTORI,[a] TERUAKI AKAO,[b] KYOICHI KOBASHI,[b] KATSUMI KAGEI,[c] KEIICHI FUKUYAMA,[c] TOMITAKE TSUKIHARA[c] and TSUNEO NAMBA*,[a]

Research Institute for Wakan-Yaku (Oriental Medicines),[a] Faculty of Pharmaceutical Sciences,[b] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan and Faculty of Engineering, Tottori University,[c] 101 Minami 4-chome, Koyama-cho, Tottori 680, Japan

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Paeoniflorin from peony roots was incubated with a cell suspension of *Bacteroides fragilis* or *Lactobacillus brevis* isolated from human intestinal flora. Paeoniflorin was converted into the 7*S* and 7*R* isomers of paeonimetaboline I as major metabolites, along with the 7*R* and 7*S* isomers of paeonimetaboline II as minor metabolites in the case of the former strain. The structures were elucidated by various spectroscopic methods, and the structure of 7*R*-paeonimetaboline I was confirmed by X-ray analysis.

Keywords——*Bacteroides fragilis*; biotransformation; 5-hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran; intestinal bacteria; *Lactobacillus brevis*; *Paeonia albiflora*; paeoniflorin; paeonimetaboline I; paeonimetaboline II; X-ray analysis

In our preceding papers,[2,3] we reported that paeoniflorin (**1**), as well as oxypaeoniflorin and benzoylpaeoniflorin, isolated from peony roots, was converted into paeonimetabolines by human intestinal bacteria. The structure of a major metabolite was concluded to be 7*S*-paeonimetaboline I (**2**). Similarly, albiflorin, a minor constituent of the peony roots, was metabolized to paeonilactones A (**10**) and B.

In order to prepare a sufficient amount of paeonimetaboline I for further study, we again surveyed various bacterial strains from human feces for ability to transform **1** to **2** during a short period of incubation, and found that some bacterial strains such as *Bacteroides fragilis* and *Lactobacillus brevis* have potent transforming activity, which had not been noticed in the previous screening experiments because of the long period of incubation used (the metabolites decreased in amount during prolonged incubation and almost completely disappeared within 16 h).[2]

In the present paper, we report the isolation of the new 7*R*-epimer (**3**) of paeonimetaboline I and a mixture of 7*R* and 7*S* isomers of paeonimetaboline II (**4a** and **4b**), and the elucidation of the structures of these metabolites by various spectroscopic methods.

Materials and Methods

Instruments——Melting points (mp) were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a Hitachi 260-10 infrared spectrophotometer. Proton and carbon-13 nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were measured with JEOL JNM-GX 400 (^{1}H, 400 MHz), JEOL FX-270 (^{1}H, 270 MHz) and JEOL-FX 90Q (^{1}H, 90 MHz; ^{13}C, 22.5 MHz) NMR spectrometers.

In ^{13}C-NMR spectra, the multiplicities were determined on the basis of the off-resonance decoupling (OFR) and insensitive nuclei enhanced by polarization transfer (INEPT) techniques. Tetramethylsilane was used as an internal standard in all the measurements. Mass spectra (MS) were measured with a JEOL JMS D-200 mass spectrometer at an ionization voltage of 70 eV. Specific rotations were taken on a Jasco model DIP-4 automatic polarimeter. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-210A spectrophotometer. High-performance liquid chromatography (HPLC) was carried out on a Tri-Rotar-V equipped with a UVIDEC-100-V detector (JASCO) using a column (250 mm × 4.6 mm i.d.) of Chemopak, Nucleosil 50-5 (5 μ, Chemco Co. Ltd., Osaka).

Chromatography of Metabolites——Wakogel C-200 was used for column chromatography. Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} or Merck PSC-60 F_{254} (preparative) plates with a solvent system of $CHCl_3$–MeOH–benzene (5:1:1). Spots on the plates were visualized by exposure to iodine vapor or by spraying with an anisaldehyde–H_2SO_4 reagent, followed by heating.

Metabolism of 1 by *Lactobacillus brevis*——A precultured bacterial suspension (500 ml) of *L. brevis* was added to GAM broth (4.5 l) and cultivated for 12 h at 37 °C under anaerobic conditions. The culture was centrifuged at 7000 rpm for 10 min. The precipitates were washed with saline solution, centrifuged, and suspended in 0.1 M phosphate buffer (625 ml). The suspension was transferred into five tubes. Compound **1** (600 mg/10 ml in the same buffer) was then added portionwise into each tube and was anaerobically incubated for 4 h at 37 °C. The mixture was extracted three times with ethyl acetate (AcOEt, 200 ml each) and the organic layer was concentrated *in vacuo* to give an oily residue. The combined residues (0.3 g) were applied to a column of silica gel (40 g, 19 × 240 mm). The column was thoroughly washed with benzene and eluted with benzene–$CHCl_3$ (1 : 1). Fractions (50 ml each) were collected and monitored by silica gel TLC and ^{1}H-NMR spectroscopy. Fractions 1—5 afforded a colorless oil, (7*S*-paeonimetaboline I, **2**, 26 mg, 11%)[2] and fractions 11—15 yielded a crystalline compound (23 mg, 9.6%), which gave pure crystals from hexane–$CHCl_3$ (9 : 1) (**3**, 11 mg) on recrystallization. Fractions 6—10 gave a mixture of **2** and **3** (21 mg, 8.8%).

7*S*-Paeonimetaboline I (2)——The physical properties were reported in the previous paper.[2]

7*R*-Paeonimetaboline I (3)——Colorless prisms, mp 146-148 °C. High resolution MS: Found, 198.0853; Calcd for M^+, $C_{10}H_{14}O_4$, 198.0892. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1705 (C=O). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.90 (3H, d, J=7.3 Hz, 8-H_3), 1.29 (3H, s, 10-H_3), 2.07 (1H, dq, J=7.5, 7.3 Hz, 7-H), 2.15 and 2.35 (each 1H, dd, J=13.4, 2.3 Hz; J=13.4, 3.4 Hz, 5-H_2), 2.60 and 2.64 (2H, ABq, J=17.7 Hz, 2-H_2), 2.65 (1H, m, 4-H), 5.14 (1H, br s, 9-H). MS m/z: 198 (M^+), 180 (M^+-H_2O), 152, 124, 109, 98, 83, 69 (base peak), 55. ^{13}C-NMR: see Table I.

Metabolism of 1 by *Bacteroides fragilis* ss. *thetaotus*——Compound **1** (2.1 g) was incubated with *B. fragilis* ss. *thetaotus* under conditions similar to those described above. After extraction with AcOEt, the organic layer was evaporated *in vacuo* to give an oily residue (0.9 g). The residue was chromatographed on silica gel (80 g; column size, 19 × 350 mm). The column was washed with benzene and eluted with benzene–$CHCl_3$ (1 : 1). Fractions were collected (60 ml/flask). Fractions 32—41, 42—49 and 50—61 afforded **2** (colorless oil, 105 mg, 12.6%), a mixture of **2** and **3** (oil, 103 mg, 12.3%) and **3** (prisms, 52 mg, 6.2%), respectively. Another oily substance (8 mg, 0.9%) was obtained from a $CHCl_3$ eluate; this was identical with paeonimetaboline II (**4**).[2]

X-Ray Analysis of 3——The unit-cell constants and intensities were measured at room temperature using Ni-filtered CuK_α radiation on a Rigaku four-circle diffractometer. Crystal data are as follows: $C_{10}H_{14}O_4$, M_r=198.1, orthorhombic, space group $P2_12_12_1$, a=11.727(3), b=7.463(2), c=10.852(3) Å, V=949.8 Å^3, Z=4, D_c=1.385 g·cm^{-3}. The θ–2θ scan technique was applied with scan width of 1.1° ±0.15° tanθ. The backgrounds were counted for 4 s on both sides of the scan range. Of the 877 independent reflections measured up to 2θ=123°, 851 reflections with $F>3\sigma(F)$ were used for the structure analysis. Periodically monitored reflections showed no significant change in intensity. The intensities were corrected for Lorentz and polarization factors. The structure was solved by the direct method.[4] The positional and anisotropic thermal parameters were refined by the block-diagonal least-squares method.[5] The hydrogen atoms were located from a difference Fourier synthesis, and included in the successive refinements with isotropic temperature factors. Extinction correction was made for the eight strongest reflections. The unit weight was applied for all reflections. The final R value was 0.041 for 851 significant reflections. The atomic scattering factors were taken from the International Tables for X-Ray Crystallography.[6]

Paeonimetaboline II (4)——Epimeric mixture consisting of **4a** (33%) and **4b** (67%). The following assignments of ^{1}H-NMR signals ($CDCl_3$, 400 MHz) were made on the basis of the peak intensities of paired signals. **4a**: δ: 1.12 (3H, d, J=6.4 Hz, 8-H_3), 1.36 (3H, s, 10-H_3), 2.02 (1H, ddq, J=14.5, 8.0, 6.5 Hz, 7-H), 2.25 and 2.29 (2H, ABq, J=12.8 Hz, 2-H_2), *ca.* 2.27 (1H, m, overlapped, 4-H), 2.41 and 2.85 (each 1H, d and dd, J=14.5 Hz; J=14.5, 7.4 Hz, 5-H_2), 3.65 and 4.05 (each 1H, dd, J=16.8, 7.8 Hz; J=16.8, 8.0 Hz, 9-H_2). **4b**: δ: 1.13 (3H, d, J=6.4 Hz, 8-H_3), 1.33 (3H, s, 10-H_3), 2.15 (1H, m, 7-H), 2.32 and 2.38 (2H, ABq, J=13.2 Hz, 2-H_2), *ca.* 2.38 (1H, m, overlapped, 4-H), 2.37 and 2.72 (each 1H, d and dd, J=14.0 Hz; J=14.0, 7.0 Hz, 5-H_2), 3.67 and 4.08 (each 1H, dd, J=16.8, 9.2 Hz; J=16.8, 8.7 Hz, 9-H_2).

7*S*-Paeonimetaboline-6-*O*-3,5-dinitrophenyl Carbamate (5)——3,5-Dinitrophenyl isocyanate (25 mg, Sumitomo Chem. Co. Ltd., Osaka) and dry pyridine (0.05 ml) were added to a solution of **2** (3.0 mg) in dry toluene (0.5 ml). The mixture was kept overnight at 37 °C, and then cooled. Methanol (2 ml) was added and the solvent was removed *in vacuo*. The product was subjected to preparative silica gel TLC to give a pure carbamate (**5**, *ca.* 2 mg) as a colorless oil.

^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.18 (3H, d, J=7.3 Hz, 8-H_3), 1.37 (3H, s, 10-H_3), 1.99 (1H, brq, J=7.3 Hz, 7-H), 2.56 (1H, m, 4-H), 2.71 and 2.79 (each 1H, dd, J=13.6, 3.4 Hz; J=13.6, 2.7 Hz, 5-H_2), 2.81 and 3.28 (each 1H, d, J=17.3 Hz, 2-H_2), 5.31 (1H, brs, 9-H), 8.67 (2H, d, J=1.9 Hz, 2'-, 6'-H), 8.78 (1H, t, J=1.9 Hz, 4'-H).

7*R*-Paeonimetaboline-6-*O*-3,5-dinitrophenyl Carbamate (6)——Under conditions similar to those described above, **3** was reacted with 3,5-dinitrophenyl isocyanate to yield **6** as a white powder. ^{1}H-NMR ($CDCl_3$, 270 MNz) δ: 0.93 (3H, d, J=7.3 Hz, 8-H_3), 1.37 (3H, s, 10-H_3), 2.18 (1H, dq, J=7.5, 7.3 Hz, 7-H), 2.61 and 2.85 (each 1H, dd, J=13.2, 3.3 Hz; J=13.2, 2.3 Hz, 5-H_2), 2.71 (1H, m, 4-H), 2.75 and 3.14 (each 1H, d, J=17.6 Hz, 2-H_2), 5.32 (1H, brs, 9-H), 8.67 (2H, d, J=1.9 Hz, 2'-, 6'-H), 8.78 (1H, t, J=1.9 Hz, 4'-H).

5-Hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran (7)——Colorless oil; $[\alpha]_D^{24}$: 0 (c=0.22, MeOH). High resolution MS: Found 164.0847; Calcd for M^+, $C_{10}H_{12}O_2$, 164.0837. MS m/z: 164 (M^+), 159 ($M^+ - CH_3$), 121, 91 (base peak), 77. UV λ_{max}^{EtOH} nm (log ε): 319 (2.70), 313 sh (2.65). IR ν_{max}^{KBr} cm^{-1}: 3360, 1458, 1165, 865. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.21 (3H, d, J=7.0 Hz, 3-Me), 2.12 (3H, s, 6-Me), 3.41 (1H, br sextet, J=7.4 Hz, 3-H), 3.94 (1H, dd, J=8.7, 7.4 Hz, 2-H_a), 4.52 (1H, dd, J=8.7, 8.5 Hz, 2-H_b), 6.49 and 6.54 (each 1H, s, 4-H, 7-H). ^{13}C-NMR ($CDCl_3$, 22.5 MHz) δ: 16.0 (q, 3-Me), 19.1 (q, 6-Me), 36.8 (d, C-3), 78.3 (t, C-2), 110.0 (d), 110.8 (d), 122.9 (s, C-6), 130.6 (s), 147.7 (s), 153.6 (s).

5-Acetoxy-3,6-dimethyl-2,3-dihydrobenzofuran (8)——On acetylation of **7** (4 mg) with acetic anhydride (0.3 ml) in pyridine (0.3 ml), a colorless oil (**8**, 2 mg) was obtained. MS m/z: 206 (M^+), 164, 149 (base peak), 121, 83, 73, 57, 55. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.22 (3H, d, J=6.9 Hz, 3-Me), 2.04 (3H, s, 6-Me), 2.22 (3H, s, 5-OAc), 3.45 (1H, br sextet, J=7.5 Hz, 3-H), 3.99 (1H, dd, J=8.6, 7.6 Hz, 2-H_a), 4.60 (1H, dd, J=8.6, 8.4 Hz, 2-H_b), 6.55 (1H, s, 7-H), 6.71 (1H, s, 4-H).

Results

Metabolism of Paeoniflorin (1) by *Bacteroides fragilis* ss. *thetaotus* and *Lactobacillus brevis*

Through a 4 h anaerobic incubation with a bacterial suspension of either *B. fragilis* or *L. brevis*, **1** was converted to two major metabolites (**2** and **3**), which were not well separated by TLC, together with two minor metabolites (**4a** and **4b**) in the case of the former bacterium. These metabolites decreased in amount during prolonged incubation and disappeared almost completely in 16 h under the incubation conditions used. *Bacteroides fragilis* seemed to grow more rapidly and to have stronger metabolizing activity than *L. brevis*.

Structures of Metabolites

The first major metabolite (Rf=0.52 on TLC) was identified by direct spectroscopic comparisons as 7*S*-paeonimetaboline I (**2**) which had previously been obtained by incubation of **1** with a mixture of bacteria from human feces.

The second major metabolite, colorless prisms, mp 146—148 °C, had almost the same Rf value with as **2**, but could be separated by repeated column chromatography. The chemical composition was determined to be $C_{10}H_{14}O_4$, isomeric with **2**, by high-resolution mass spectrometry. The compound showed no UV absorption, but there were characteristic strong IR bands assignable to a hydroxyl (3420 cm^{-1}) and a ketonic carbonyl (1705 cm^{-1}) in a six-

1

2 : R=-H
5 : R=-CO-NH-C_6H_3-$(NO_2)_2$

3 : R_1=-H, R_2=-H
6 : R_1=-CO-NH-C_6H_3-$(NO_2)_2$, R_2=-H
9 : R_1=-H, R_2=-O-CO-C_6H_5

4

10

7 : R=-H
8 : R=-CO-CH_3

Chart 1. Structures of Paeoniflorin, Paeonimetabolines and Related Compounds

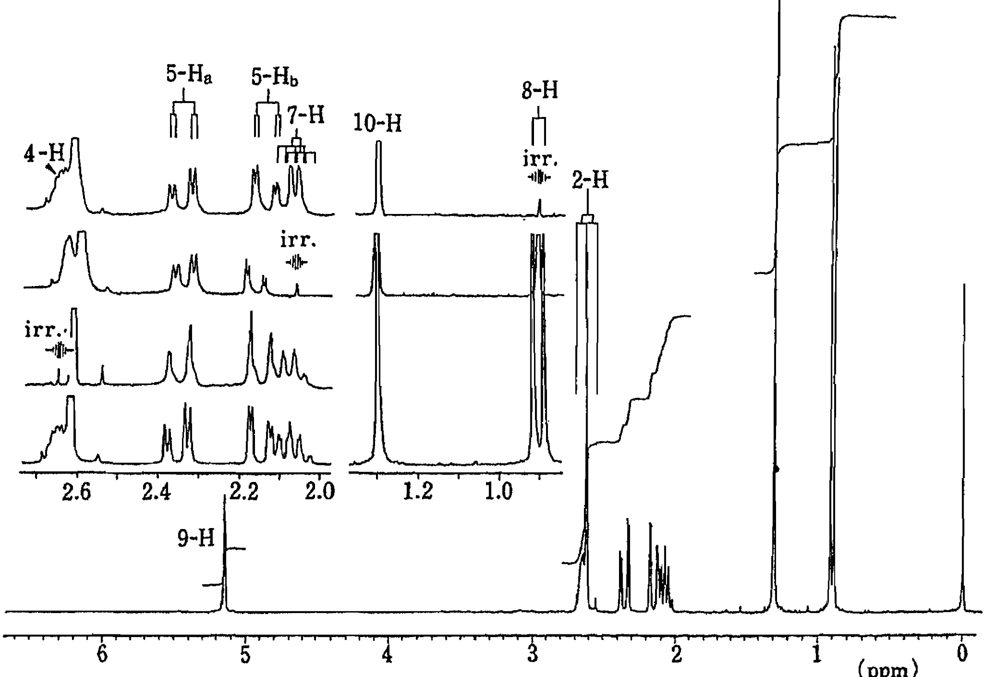

Fig. 1. ^{1}H-NMR Spectrum of 7*R*-Paeonimetaboline I (3) Measured at 270 MHz

TABLE I. ^{13}C-NMR Data for Paeonimetabolines and Related Compounds

Carbon No.	Compounds				
	2	**3**	**4a**$^{a)}$	**4b**$^{a)}$	**10**
C-1	77.4 (s)	78.3 (s)	72.8 (s)	72.8 (s)	73.6 (s)
C-2	47.5 (t)	46.8 (t)	38.6 (t)	38.8 (t)	35.5 (t)
C-3	210.9 (s)	210.1 (s)	104.8 (s)	104.8 (s)	73.5 (d)
C-4	50.2 (d)	49.6 (d)	52.3 (d)	53.4 (d)	37.9 (d)
C-5	30.4 (t)	34.3 (t)	47.5 (t)	47.1 (t)	42.1 (t)
C-6	101.6 (s)	101.3 (s)	212.7 (s)	212.7 (s)	210.4 (s)
C-7	37.9 (d)	38.4 (d)	40.2 (d)	42.1 (d)	43.8 (d)
C-8	14.6 (q)	13.3 (q)	16.3 (q)	15.7 (q)	13.1 (q)
C-9	103.1 (d)	103.0 (d)	72.4 (t)	73.4 (t)	177.0 (s)
C-10	20.9 (q)	21.1 (q)	25.2 (q)	25.6 (q)	24.8 (q)

a) 4a and 4b were not separated, but their ^{13}C-NMR chemical shifts were deduced on the basis of the relative signal intensities. Abbreviations given in parentheses indicate the signal patterns based on OFR and INEPT experiments. s, singlet; d, doublet; t, triplet; q, quartet.

membered ring. The ^{1}H-NMR (Fig. 1) and ^{13}C-NMR (Table I) spectra showed the presence of identical functional groups with those of **2**; a *sec*-methyl (^{1}H; δ 0.90; ^{13}C, δ 13.3), a *tert*-methyl (^{1}H, δ 1.29; ^{13}C, δ 21.1), an acetal (^{1}H, δ 5.14; ^{13}C, δ 103.0), a hemiketal (^{13}C, δ 101.3), a ketonic carbonyl (^{13}C, δ 210.1), *etc.* The ^{1}H-double resonance experiments (Fig. 1) indicated the following spin correlations: a methine proton at δ 2.65 (m, 4-H) *versus* methylene protons at δ 2.15 and 2.35 (each dd, 5-H_a and 5-H_b) or a methine proton at δ 2.07 (dq, 7-H); a methine proton (7-H) *versus* methyl protons (8-H_3) at δ 0.90. These findings suggested that **3** had the same skeleton as **2**, which was further supported by the evidence that most of the ^{13}C-signals of **3** were quite similar to those of **2**, except the C-8 and C-5 signals. In contrast with the case

of **2**, in which γ-gauche steric interaction is apparent between the C-8 and C-5 carbons, no appreciable neighboring group effect was observed in **3**, based on a comparison of the signals with the corresponding signals of paeoniflorigenone (**9**).[7] This suggests that the C-8 methyl group is projected just above the C-3 carbonyl group (7*R*-configuration), as in **9**. This conclusion is further supported by the observation of an upfield shift (*ca.* 0.22 ppm unit) of the C-8 methyl proton signal in **3** compared to that of **2** in the ^{1}H-NMR spectrum. Based on the above evidence, **3** was deduced to be 7*R*-paeonimetaboline I (Chart 1).

The structure and stereochemistry were finally established by a single-crystal X-ray analysis using the direct method. A perspective view of the molecule drawn by DCMS-3[8] is shown in Fig. 2. The atomic parameters,[9] bond lengths and bond angles are listed in Tables II and III.

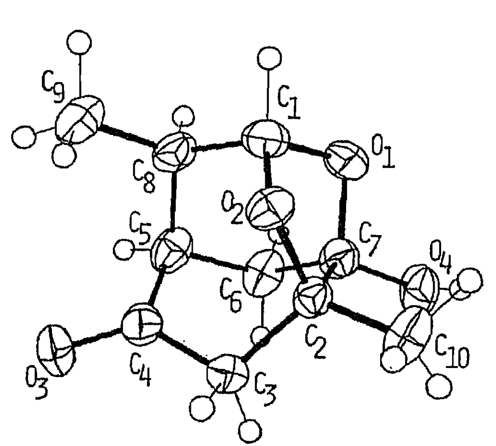

Fig. 2. Perspective View of 7*R*-Paeonimetaboline I (3)

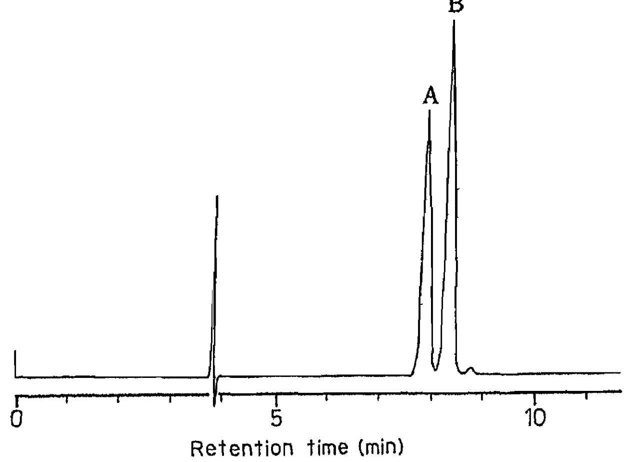

Fig. 3. HPLC Elution Profile of Carbamates of the 7*R* and 7*S* Epimers of Paeonimetaboline I

Conditions: column, Nucleosil 50-5 (5 μ, 4.6 mm i.d. × 25 cm); mobile phase, *n*-hexane–1,2-dichloroethane–ethanol (20 : 4 : 1); flow rate, 1.3 ml/min; pressure, 30 kg/cm^2; detection, UV at 250 nm. Peaks A and B are the 3,5-dinitrophenylcarbamates of **2** and **3**, respectively.

TABLE II. Final Atomic Coordinates of 7*R*-Paeonimetaboline I (3)

Non-hydrogen atoms	*x*	*y*	*z*	Hydrogen atoms	*x*	*y*	*z*
C(1)	0.3634 (4)	0.6206 (6)	0.3405 (4)	H(1)	0.406 (4)	0.613 (6)	0.243 (4)
C(2)	0.3976 (3)	0.5368 (5)	0.5412 (3)	H(2)	0.478 (4)	0.725 (6)	0.666 (4)
C(3)	0.3914 (4)	0.6883 (6)	0.6361 (4)	H(3)	0.359 (4)	0.649 (7)	0.713 (4)
C(4)	0.3342 (4)	0.8491 (6)	0.5783 (4)	H(4)	0.174 (4)	0.913 (6)	0.483 (4)
C(5)	0.2428 (4)	0.8062 (6)	0.4834 (4)	H(5)	0.153 (4)	0.632 (6)	0.610 (4)
C(6)	0.1881 (3)	0.6262 (6)	0.5178 (4)	H(6)	0.116 (4)	0.596 (6)	0.462 (4)
C(7)	0.2752 (3)	0.4806 (6)	0.4956 (4)	H(7)	0.227 (4)	0.786 (7)	0.295 (4)
C(8)	0.2945 (4)	0.7940 (6)	0.3519 (4)	H(8)	0.452 (4)	0.961 (7)	0.365 (4)
C(9)	0.3670 (5)	0.9562 (7)	0.3150 (4)	H(9)	0.328 (4)	1.069 (7)	0.333 (4)
C(10)	0.4698 (4)	0.3824 (6)	0.5846 (5)	H(10)	0.388 (4)	0.954 (7)	0.220 (5)
O(1)	0.2943 (3)	0.4689 (4)	0.3641 (2)	H(11)	0.553 (4)	0.419 (6)	0.602 (4)
O(2)	0.4501 (2)	0.6118 (4)	0.4317 (2)	H(12)	0.478 (4)	0.283 (7)	0.518 (5)
O(3)	0.3584 (3)	0.9998 (4)	0.6086 (3)	H(13)	0.452 (4)	0.328 (7)	0.647 (4)
O(4)	0.2330 (2)	0.3200 (4)	0.5396 (3)	H(14)	0.283 (4)	0.251 (6)	0.537 (4)

TABLE III. Bond Lengths (Å) and Angles (°) for Non-hydrogen Atoms of 7*R*-Paeonimetaboline I (3)

C(1)–C(8)	1.530 (6)	C(1)–O(1)	1.416 (6)	C(1)–O(2)	1.420 (5)
C(2)–C(3)	1.532 (6)	C(2)–C(7)	1.576 (6)	C(2)–C(10)	1.506 (6)
C(2)–O(2)	1.450 (5)	C(3)–C(4)	1.512 (6)	C(4)–C(5)	1.520 (6)
C(4)–O(3)	1.206 (6)	C(5)–C(6)	1.535 (6)	C(5)–C(8)	1.553 (6)
C(6)–C(7)	1.510 (6)	C(7)–O(1)	1.447 (5)	C(7)–O(4)	1.382 (5)
C(8)–C(9)	1.532 (7)				

C(8)–C(1)–O(1)	111.1 (4)	C(8)–C(1)–O(2)	111.1 (4)
O(1)–C(1)–O(2)	104.3 (3)	C(3)–C(2)–C(7)	111.4 (3)
C(3)–C(2)–C(10)	112.4 (4)	C(3)–C(2)–O(2)	106.6 (3)
C(7)–C(2)–C(10)	114.0 (3)	C(7)–C(2)–O(2)	103.4 (3)
C(10)–C(2)–O(2)	108.3 (3)	C(2)–C(3)–C(4)	109.2 (4)
C(3)–C(4)–C(5)	115.3 (4)	C(3)–C(4)–O(3)	121.5 (4)
C(5)–C(4)–O(3)	123.2 (4)	C(4)–C(5)–C(6)	108.3 (4)
C(4)–C(5)–C(8)	111.1 (4)	C(6)–C(5)–C(8)	109.6 (4)
C(5)–C(6)–C(7)	108.0 (4)	C(2)–C(7)–C(6)	112.0 (3)
C(2)–C(7)–O(1)	100.6 (3)	C(2)–C(7)–O(4)	116.6 (3)
C(6)–C(7)–O(1)	107.8 (3)	C(6)–C(7)–O(4)	109.1 (3)
O(1)–C(7)–O(4)	110.1 (3)	C(1)–C(8)–C(5)	109.2 (4)
C(1)–C(8)–C(9)	110.8 (4)	C(5)–C(8)–C(9)	114.2 (4)
C(1)–O(1)–C(7)	102.6 (3)	C(1)–O(2)–C(2)	106.6 (3)

Despite many attempts, we could not quantitatively analyze the two epimers by TLC-densitometry or GC-MS after derivatization (silylation), but the 3,5-dinitrophenylcarbamates of **2** and **3** were well separated with high sensitivity by normal phase HPLC. Figure 3 shows a chromatogram of the mixture; peaks A and B were assigned to the 3,5-dinitrophenylcarbamates of **2** and **3**, respectively.

The minor metabolites ($Rf=0.42$) were obtained as a colorless oil, identical with paeonimetaboline II, as had been reported in the previous paper.[2] The oil, however, was a mixture of isomers (**4a** and **4b**) in a ratio of 2:1 on the basis of 400 MHz ^{1}H-NMR analysis. Since various attempts to separate the two isomers were unsuccessful due to their extreme instability, the mixture was directly analyzed.

The ^{1}H-NMR and ^{13}C-NMR spectra (Table I) showed pairs of signals with different intensities, which may be assignable to *sec*-and *tert*-methyls, three methylenes including an oxymethylene, two methines including a methine adjacent to the *sec*-methyl, and hemiketal and ketonic carbons, but they did not exhibit either characteristic signals of glucose and benzoyl moieties present in the original substrate (**1**) or those of an acetal group present in the major metabolites (**2** and **3**). In addition, a significant difference in chemical shift (1.9 ppm) was only observed for the methyl-bearing carbon (C-7) between **4a** and **4b**, suggesting a mixture of 7*R* and 7*S* epimers. Based on the above data and the chemical finding that the mixture were readily decomposed to 5-hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran (**7**), as will be mentioned later, the structures of the epimers were proposed to be **4**[10] in Chart 1.

When kept in $CHCl_3$–MeOH for several days, the mixture of **4a** and **4b** decomposed to several products with UV absorption. The major product had *Rf* 0.71, but was readily transformed to another compound (**7**) with *Rf* 0.59. The isolation of the former compound was unsuccessful because of its extreme instability during purification, but the latter (**7**) was obtained as an oil with the molecular formula $C_{10}H_{12}O_2$, which afforded a monoacetate on acetylation. The UV spectrum (λ_{max} 319 nm, λ_{sh} 313 nm) showed the presence of a *p*-hydroquinone system[11] and the IR spectrum showed a strong absorption band due to a hydroxyl group. The ^{1}H-NMR and ^{13}C-NMR spectral evidence finally led to the structure of

5-hydroxy-3,6-dimethyl-2,3-dihydrobenzofuran for **7**.

Discussion

By anaerobic incubation with *B. fragilis* and *L. brevis*, paeoniflorin (**1**) was converted into 7*S* and 7*R* isomers of paeonimetaboline I (**2** and **3**, respectively), as well as those of paeonimetaboline II (**4a** and **4b**) in the case of the former bacterium. In the transformation of **1** by the two bacterial strains, the newly isolated metabolite, **3**, seems to be formed through metabolic processes analogous to those involved in the case of **2**, as reported in the preceding paper (Chart 2);[2] enzymic hydrolysis of glucosyl and benzoyl groups is considered to be the first step, followed by cleavages of the hemiketal–acetal linkage and the four-membered ring (i and ii), then formation of an aldehyde intermediate (iii) and cyclization to yield a hemiketal–acetal derivative (iv). Non-stereospecific reduction of iv leads to a mixture of the 7*R* and 7*S* isomers (**3** and **2**). Since no conversion from **2**/**3** to **4a**/**4b**, or *vice versa*, could be demonstrated during anaerobic incubation with either *B. fragilis* of *L. brevis*, the formation of **4** proceeds *via* an aldehyde intermediate (iii) and its reduced product (v). The alcohol (v) might cyclize to form a hemiketal at the C-3 position (vi), followed by non-stereospecific reduction of the terminal double bond to yield the 7*R* and 7*S* isomers of paeonimetaboline II (**4a** and **4b**). This mixture was readily dehydrated, followed by aromatization to yield a dihydrobenzofuran derivative (**7**).

7*S*-Paeonimetaboline I (**2**) has an appreciable suppressing effect on convulsions induced by pentylenetetrazole in rats.[12] Further studies are in progress to examine the difference in biological potency between the two epimers.

2 : R_1=H, R_2=CH_3
3 : R_1=CH_3, R_2=H

Chart 2. Possible Metabolic Processes of Paeoniflorin (**1**) by Intestinal Bacteria

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Separation and Determination of Fluoride Ion by Using Ion Exchange Resin Loaded with Alizarin Fluorine Blue

MASAHIKO CHIKUMA,[a] YOSHITO OKABAYASHI,[*,b] TERUMICHI NAKAGAWA,[b] AIKO INOUE[b] and HISASHI TANAKA[b]

Osaka University of Pharmaceutical Sciences,[a] Kawai, Matsubara, Osaka 580, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Sakyo-ku, Kyoto 606, Japan

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A new functional resin for the selective collection of fluoride ion was prepared from anion-exchange resin and lanthanum complex of Alizarin Fluorine Blue (AFB–La). The resin loaded with AFB–La was effective for the selective adsorption of fluoride ion in batch and column operations. Fluoride ion adsorbed could be eluted completely with a small volume of 1 M sodium hydroxide, and the concentration of fluoride ion thus concentrated in the eluate was determined by using an ion selective electrode. The resin could be regenerated by washing with water and used repeatedly without functional deterioration. A satisfactory result was obtained by the use of this resin in the determination of fluoride ion present at concentrations lower than that which can be determined directly by the ion selective electrode method. The interference caused by common cations and anions was not serious except for Al^{3+} and Fe^{3+}

Keywords——fluoride ion; ion-exchange resin; functionalized resin; alizarin fluorine blue; lanthanum complex; separation; preconcentration; determination; ion selective electrode; tap water

Introduction

Fluorine has recently been recognized as a nutritionally essential element.[1] The comparatively narrow concentration gap between the nutritional level and toxic level demands the development of the methods for the accurate determination of low levels.[2] An effective method for the separation of fluoride ion in place of the conventional microdifusion method[3,4] which is complicated in operation, is desirable in various fields such as studies on dental diseases and on the metabolism and pharmacokinetics of fluorinated drugs which release trace amount of fluoride ion.[5] Very low level of fluoride ion encountered in these cases can not be determined accurately by the direct use of an ion selective electrode without preconcentration. In Japan, the concentration of fluoride ion in waste water is required to be below 15 ppm by the Water Pollution Prevention Act. The precipitation method with calcium chloride has been used generally for the removal of fluoride ion from waste water. However, this method is not always satisfactory in relation to the above requirement, because the solubility of calcium fluoride is not sufficiently low.[6] Accordingly, the development of an effective method for the final removal of fluoride ion is necessary in waste water treatment. We have attempted to develop a functional resin for the selective collection of fluoride ion to offer

Fig. 1. Alizarin Fluorine Blue (AFB)

an effective method to meet the demands mentioned above. This paper deals with a functional resin prepared from anion-exchange resin and alizarin fluorine blue (AFB, Fig. 1).[7-9]

Experimental

Reagents——A standard solution of fluoride (1000 ppm as F^-) was prepared by dissolving 2.210 g of sodium fluoride, which had been dried at 200 °C for 2 h, in 1000 ml of deionized, distilled water. The solution was stored in a polypropylene bottle. Amberlite CG-400 (IRA-400 type, 100—200 mesh, 8% divinyl benzene) of nitrate form was used as an anion-exchange resin, AFB and Alfusone (AFB–La chelate+buffer) were purchased from Dojindo Laboratories (Kumamoto, Japan). Lanthanum ion solution (0.02 M) was prepared from $La(NO_3)_3 \cdot 6H_2O$ (Wako Pure Chemicals Ltd.). All reagents used were of analytical reagent grade quality.

Instruments——The determination of fluoride ion was carried out with an Orion Ion Meter 901 equipped with an Orion model 94-09 fluoride ion selective electrode and a reference electrode, and the total ionic strength adjustment buffer[10,11] consisting of cyclohexanediaminetetraacetic acid, acetic acid, sodium hydroxide and sodium chloride was used. The pH of the solution was measured with a TOA model HM-20E pH meter and a combined glass electrode.

Preparation of Resin Loaded with AFB–La——AFB (15.4 mg) was dissolved in 10 ml of 0.5 M sodium hydroxide, and the solution was diluted to 200 ml with distilled water. The pH of the solution was adjusted to about 5 by addition of 0.2 M acetic acid. Then 2 ml of 0.02 M lanthanum ion solution was added to the AFB solution, and the mixture was shaken for about 2 h at 30 °C. The anion-exchange resin(1 g) was added to the AFB–La complex solution, and the mixture was shaken for 5 h at 30 °C. The resin loaded with AFB–La complex (AFB–La resin) was collected on a filtration funnel, washed with water and methanol, and air-dried.

Batch Operation——AFB–La resin (25—1200 mg) was added to buffer solution (acetate, phthalate or succinate) containing various known concentrations of fluoride ion. Acetone and foreign ions were contained in the solution of fluoride ion when their effects were to be examined. The mixture was shaken for 3—4 h at 20—40 °C. Then the resin was filtered off, and fluoride ion remaining in the filtrate was determined by using the ion selective electrode.

Column Operation——A polypropylene column (6 to 10 mm i.d.) was filled with AFB–La resin (0—40 μmol/g-resin). The length of the resin zone was 3—11 cm. Sample solution containing various known concentrations of fluoride ion and sodium chloride (0—0.4 M) in acetate buffer (pH 4.2) was applied to the column at a constant flow rate by using a peristaltic pump. The effluent was collected with a fraction collector, and fluoride ion in each fraction was determined by using the ion selective electrode.

Regeneration and Preconcentration——Sample solution (50 ml) containing pH4.2 acetate buffer and 950 μg of fluoride ion was passed through a column filled with 1 g of resin loaded with 40 μmol of AFB–La at a flow rate of 0.58—1.0 ml/min. Fluoride ion adsorbed was eluted from the column with 5-10 ml of 1 M sodium hydroxide at a flow rate of 0.58—1.0 ml/min. The effluent was neutralized with acetic acid, diluted exactly to 10—50 ml with distilled water exactly and subjected to measurement of the concentration of recovered fluoride ion by using the ion-selective electrode to determine the rate of regeneration of fluoride ion. Water samples containing unknown amounts of fluoride ion were treated similarly to examine the efficiency of this method for preconcentration of fluoride ion.

Results and Discussion

Batch Operation

The optimal conditions for the collection of fluoride ion on AFB–La resin were investigated by batch operation. As shown in Fig. 2, a satisfactory result was obtained when the pH of the solution was adjusted to 3—4 with acetate buffer. The efficiency of the resin decreased with increasing pH value of the solution owing to the competitive effect of hydroxide ion and fluoride ion in the reaction with AFB–La. The release of AFB–La from the resin was observed when the pH of the solution was below 3. The effect of shaking time is shown in Fig. 3. Three or four hours were necessary to attain equilibrium.

The adsorption isotherms obtained at 20, 30 and 40 °C by batch operation in the acetate buffer solution are shown in Fig. 4. The isotherms show slight deviation from linearity in the high fluoride concentration region. The amount of fluoride ion adsorbed was slightly decreased as the temperature was raised from 20 to 40 °C. As can be seen in Fig. 4, the efficiency of AFB–La resin for the collection of fluoride ion was much higher than that of the

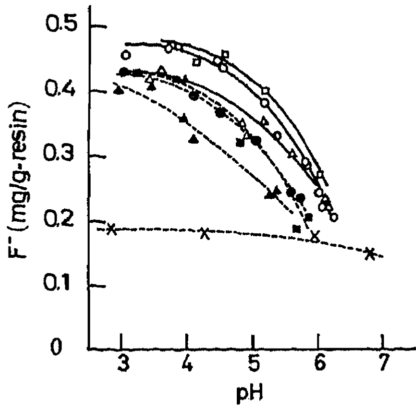


Fig. 2. Collection of Fluoride Ion from Buffer Solutions of Various pH Values

Succinic acid–hexamine: —○—, 20% acetone; —●—, 0% acetone. Potassium hydrogen phthalate–hexamine: —△—, 20% acetone; —▲—, 0% acetone. AcOH–AcONa: —□—, 20% acetone; —■—, 0% acetone. Concentration of fluoride ion: 2 ppm. Total volume: 50 ml. Amount of resin: 200 mg (Alfusone 1.1 g/g-resin). —×—, blank (CG-400 without AFB–La).

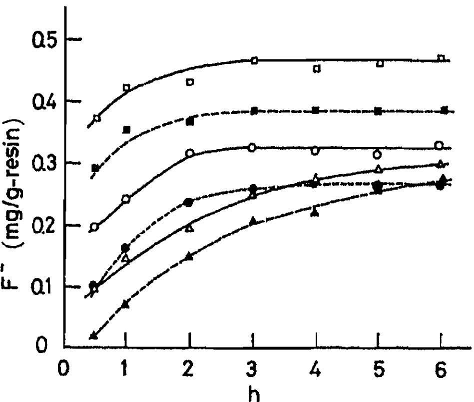


Fig. 3. Effect of Shaking Time

Succinate: —○—, 20% acetone (pH 5.6); —●—, 0% acetone (pH 4.6). Phthalate: —△—, 20% acetone (pH 5.5); —▲—, 0% acetone (pH 5.1). Acetate: —□—, 20% acetone (pH 4.6); —■—, 0% acetone (pH 4.1). Concentration of fluoride ion: 2 ppm. Total volume: 100 ml. Amount of resin: 400 mg (Alfusone 1.1 g/g-resin).

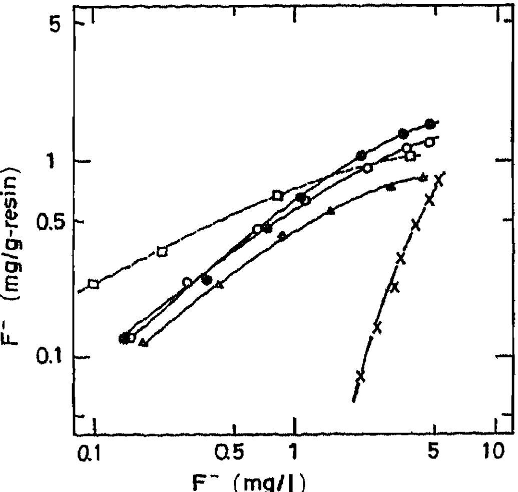


Fig. 4. Adsorption Isotherms at 20, 30 and 40 °C

AFB-La-loaded resin (AFB 40 μmol/g-resin, AFB : La = 1 : 1): —△—, 20 °C; —○—, 30 °C; —□—, 30 °C (20% acetone); —●—, 40 °C. Amberlite CG-400 without AFB–La: —×—, 30 °C, pH 4.2 (0.04 M acetate buffer), 0.1 M NaCl.

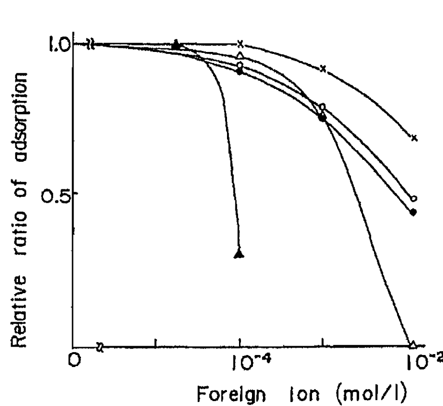


Fig. 5. Influence of Foreign Ions on Adsorption of Fluoride Ion

Shaking time, 3 h; temperature, 30 °C; amount of AFB-La-loaded resin, 200 mg; concentration of fluoride ion, 3 ppm; pH 4.2 (acetate buffer); total volume, 50 ml. —○—, Ca^{2+}; —●—, Mg^{2+}; —▲—, Al^{3+}; —△—, Fe^{3+}; —×—, NH_4^+.

untreated anion exchange resin when the concentration of fluoride ion was low. However, the efficiency of AFB–La resin did not differ much from that of the anion exchange resin when the concentration of fluoride ion high. These results suggest that the AFB–La resin has two different binding sites for the adsorption of fluoride ion, namely, nonspecific anion-exchange sites and AFB–La sites which can bind fluoride ion strongly and specifically. Chloride ion caused interference only with the adsorption of fluoride ion on the nonspecific ion exchange sites. Furthermore, higher efficiency was achieved by the addition of acetone, which enhanced

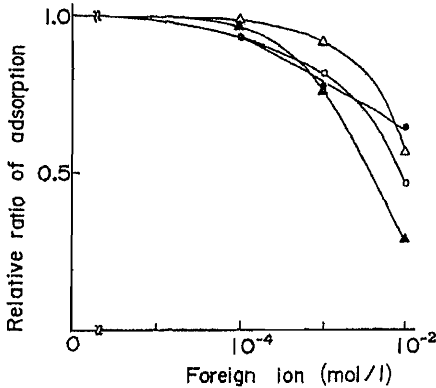


Fig. 6. Influence of Foreign Ions on Adsorption of Fluoride Ion

Shaking time, 3 h; temperature, 30 °C; amount of AFB–La-loaded resin, 200 mg; concentration of fluoride ion, 3 ppm; pH 4.2 (acetate buffer); total volume, 50 ml. —○—, NO_3^-; —●—, SO_4^{2-}; —△—, CH_3COO^-; —▲—, $H_2PO_4^-$

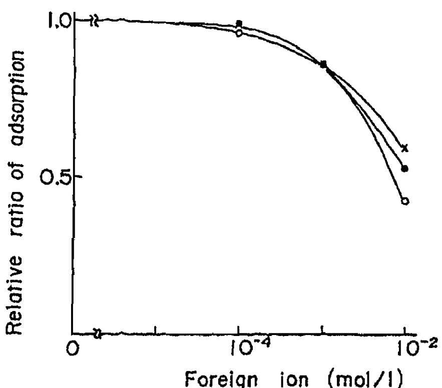


Fig. 7. Influence of Foreign Ions on Adsorption of Fluoride Ion

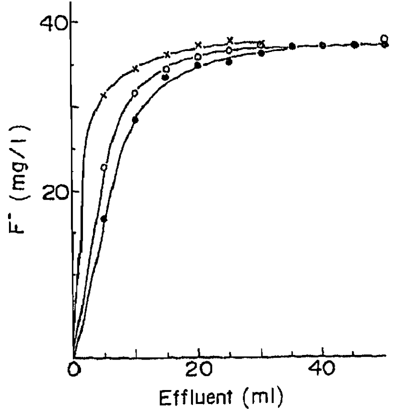
Shaking time, 3 h; temperature, 30 °C; amount of AFB–La-loaded resin, 200 mg; concentration of fluoride ion, 3 ppm; pH 4.2 (acetate buffer); total volume, 50 ml. —×—, Cl^-; —●—, Br^-; —○—, I^-

the formation of the ternary complex of AFB–La with fluoride ion because of the decreased polarity of the solution. However, we omitted the addition of acetone in the practical application of this method, because its high volatility caused some decrease in the volume of the solution and because it is hazardous.

The effects of various foreign ions added as their chloride or sodium salts on the adsorption of fluoride ion were examined by batch operation. The results for the cations are shown in Fig. 5. Slight interference caused by Ca^{2+}, Mg^{2+}, and NH_4^+ may be attributed to their comparatively weak interaction with fluoride ion. On the other hand, serious interference caused by Al^{3+} is obviously attributable to the formation of the stable fluoride complex of aluminium.[12] The interference caused by Fe^{3+} can be eliminated by reduction to Fe^{2+}. The results for the anions are shown in Figs. 6 and 7. No significant interference was observed when NO_3^-, SO_4^{2-}, $H_2PO_4^-$, CH_3COO^-, Cl^-, Br^- or I^- was present in the solution at a concentration comparable to that of fluoride ion (1.58×10^{-4} M). However, when the concentration of these ions was as high as the order of 10^{-3} M, the adsorption of fluoride ion decreased to 80—90% of that achieved in the absence of these ions because of interference at the nonspecific ion exchange sites.

Column Operation

The effect of repeated use of the resin on the recovery was examined by column operation. Fluoride ion adsorbed on the AFB–La loaded resin could be eluted by the competitive reaction with sodium hydroxide solution. The adsorption ratio was 99.4 ± 0.63% even after ten cycles, and the recovery was 98.8 ± 3.6% from the third run to the tenth run. However, in the first and second runs, slightly lower values (89.9% for the first run, 92.8% for the second run) for the recovery were obtained. This result suggests that this resin contains a certain adsorption site where fluoride ion is adsorbed strongly and can hardly be eluted with 1 M sodium hydroxide, and this site may be saturated during the first and second runs. The results also indicate that AFB–La as well as lanthanum ion is retained firmly on the resin even after ten elutions with strong alkaline solution. Even in the presence of 0.1 M sodium chloride, adsorption of fluoride ion spiked in a water sample did not decrease during repeated use. The amount of total fluoride ion adsorbed on AFB–La resin was about 750 μg/g-resin, while that adsorbed on the anion-exchange resin (CG-400) without AFB–La was only about 120 μg/g-resin.



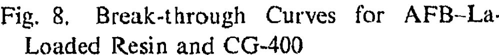


Fig. 8. Break-through Curves for AFB–La-Loaded Resin and CG-400

Column: 3 cm height, 0.6 cm i.d. Flow rate: 0.23 ml/min. Solution: pH 4.2 (acetate buffer); concentration of fluoride, 38 ppm; 0.1 M NaCl. —×—, CG-400 without AFB–La; —○—, 20 μmol/g-resin of AFB–La-loaded resin; —●—, 40 μmol/g-resin of AFB–La-loaded resin.

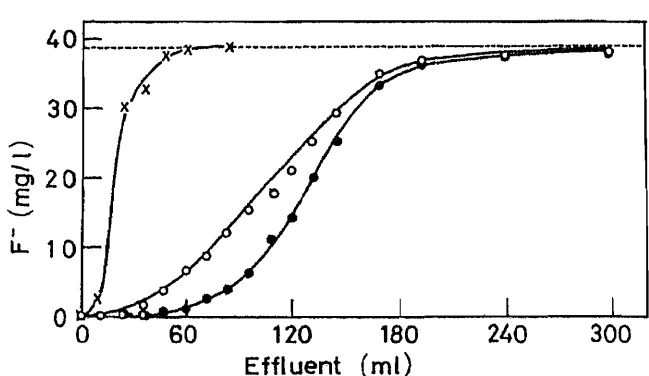


Fig. 9. Break-through Curves

Column: 11 cm height, 1 cm i.d. Amount loaded on resin: AFB–La 40 μmol/g-resin. Solution: pH 4.2 (acetate buffer); concentration of fluoride ion, 38 ppm. —○—, 0.4 M NaCl; —●—, 0.1 M NaCl; —×—, 0.4 M NaCl (CG-400 without AFB–La); -----, 38 ppm.

In order to examine the effect of the amount of AFB–La loaded on the resin, polypropylene columns (10 mm i.d.) were filled with AFB–La resin which contained various amounts of AFB–La (0, 20, 40 μmol/g-resin) and the solution containing fluoride ion (38 mg/l), 0.1 M sodium chloride and acetate buffer (pH 4.2) was applied to each column. The break-through curves for fluoride ion are shown in Fig. 8. The amount of fluoride ion adsorbed increased with increasing amount of AFB–La immobilized on the resin. This result shows that relatively high concentrations of chloride ion interfered with nonspecific adsorption of fluoride ion on the anion-exchange sites of the resin, while fluoride ion was adsorbed firmly at the AFB–La sites. The break-through curves obtained by the use of a larger column (10×110 mm) are shown in Fig. 9, which indicates that fluoride ion present in 0.4 M NaCl solution was adsorbed onto the resin loaded with AFB–La but not onto the anion-exchange resin. The adsorption capacity of this resin was not very high, as shown in Fig. 9.

The present method was applied to the preconcentration of fluoride ion in tap water supplied in Kyoto City. The concentration of fluoride ion in tap water was determined to be 0.027 ± 0.002 ppm ($n=3$). In this case, a preconcentration rate of five times was achieved and the accurate determination of low levels of fluoride ion, almost at the lower limit for direct determination by using an ion selective electrode,[13] was made possible by this method.

In conclusion, the method presented here is effective for the preconcentration of fluoride ion present at concentrations lower than the limit of direct determination by using ion selective electrode. Further, the resin presented here may be effective for the removal of fluoride in waste water treatment.

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Determination of Free and Total Phenylacetic Acid in Human and Rat Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

MASATOSHI YAMAGUCHI* and MASARU NAKAMURA

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Johnan-ku, Fukuoka 814-01, Japan

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A highly sensitive and simple high-performance liquid chromatographic method has been developed for the determination of free and total phenylacetic acid in human and rat plasma. After extraction with diethyl ether from plasma, phenylacetic acid and phenylpropionic acid (internal standard) are converted to the corresponding fluorescent derivatives by reaction with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone in the presence of potassium hydrogen carbonate and 18-crown-6 in acetonitrile. The derivatives are separated on a reversed-phase column (Radial-Pak cartridge C_{18}) with aqueous 65% (v/v) methanol and detected fluorimetrically. The detection limit for phenylacetic acid is 11 pmol/ml in plasma at a signal-to-noise ratio of 5. This sensitivity permits precise determination of free and total phenylacetic acid in 50 μl of human and rat plasma. The method was applied to the determination of free and total phenylacetic acid in plasma from healthy volunteers, and control and "behavioral despair" rats.

Keywords——phenylacetic acid; human plasma; rat plasma; high-performance liquid chromatography; fluorescence detection; 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone

Phenylacetic acid (PAA) is present as free and conjugated forms in human plasma. PAA may be mainly derived from phenylalanine and phenylethylamine by decarboxylation and deamination, and is further metabolized in the human body to its glutamine conjugate. It is indicated that the amount of PAA decreases in plasma of patients with depressive illness.[1,2] Therefore, the determination of plasma PAA in humans may be useful for the diagnosis, monitoring and investigation of depressive illness.

Recently, rats forced to swim in a restricted space have been found to be a specific animal model for depressive illness. Thus, the determination of plasma PAA in such rats may improve our understanding of depressive illness.[3]

Gas chromatography-mass spectrometric (GC-MS) methods have been most widely used for the determination of free and total (the sum of free and conjugated) PAA in human plasma.[2,4-7] Although the methods are very sensitive, they require expensive equipment and rather tedious techniques. Thus, they have not been routinely used. Recently, a simple high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection has been proposed for the determination of total PAA in human plasma.[1] However, the method has a limited sensitivity and thus requires a large amount of human plasma (2 ml). Furthermore, the method has not been applied to the determination of free PAA, which occurs in a minute amount in human plasma. No method has yet been applied to experimental small animals such as rats and mice.

We previously reported a simple and sensitive HPLC method for the simultaneous determination of free and total PAA and *p*- and *m*-hydroxyphenylacetic acids in human urine using precolumn fluorescence derivatization with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ), a fluorogenic reagent for carboxylic acids; these acids,

after extraction with diethyl ether from urine, are converted into the corresponding fluorescent compounds by reaction with Br-DMEQ and these compounds are separated on a reversed-phase (Radial Pak cartridge C_{18}) column with isocratic elution.[8] The purpose of the present research was to establish a simple, sensitive and rapid method for the determination of free and total PAA in a minute amount of human and rat plasma. The established method was used to compare free and total PAA concentrations in control rat plasma with those in plasma from rats forced to swim. Phenylpropionic acid (PPA), which is not present in human and rat physiological fluids, was used as an internal standard (IS).

Experimental

Chemicals and Solutions——All chemicals and solvents were of reagent grade, unless otherwise stated. Deionized and distilled water was used. PAA was purchased from Sigma (St. Louis, Mo., U.S.A.). Acetonitrile used for the derivatization reaction was purified as described previously.[9] Br-DMEQ was prepared as described previously;[9] it is now available from Dojindo Laboratories (Kumamoto, Japan). Br-DMEQ (1.3 mM), 18-crown-6 (3.8 mM) and PPA (3.2 μM, IS) solutions were prepared in acetonitrile. The Br-DMEQ solution could be kept for more than one week when stored in a refrigerator at 4 °C.

Apparatus and HPLC Conditions——A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow-cell operating at the excitation and emission wavelengths of 379 and 455 nm, respectively, were used. The column was a Radial Pak cartridge C_{18} (100 × 8 mm i.d.; particle size, 5 μm; Waters Assoc., Milford, Mass., U.S.A.). The mobile phase was H_2O–MeOH (35 : 65, v/v). The flow-rate was 2.0 ml/min (*ca.* 70 kg/cm^2). The column temperature was maintained at 40 ± 1 °C. This column could be used for more than 1000 injections with only a small decrease in the theoretical plate number when washed with methanol at a flow rate of 2 ml/min for *ca.* 20 min at the end of each working day. Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Plasma Samples——Male Wister rats (n = 20) weighing 210—230 g were used for the present study. The rats were housed in a well-controlled environment with free access to food and water, and were used within a day after being brought into the laboratory. The rats were divided into two groups of 10 rats each. One group (intact rats) served as a control. The other group (10 rats) was treated according to the novel forced swimming test of Porsolt *et al.*[3] Briefly, the rats were individually forced to swim once daily inside plexiglass cylinders containing water maintained at 25 °C for 15 min. After 4 daily sessions of swimming, the total duration of immobility was measured during a 5 min test. All the rats employed were judged from the forced swimming test to be in a depressive state. On the next day, the rats were killed by decapitation, and blood (2—4 ml) was collected in a centrifuge tude containing disodium ethylenediaminetetraacetate (2—4 mg) as an anticoagulant. Plasma was separated by centrifugation of the blood at 10000 *g* at 5 °C. Human plasma was obtained from fasting healthy volunteers in our laboratory. Human and rat plasma samples were stored at −40 °C until just before use.

Procedure——A 50-μl portion of plasma sample was diluted with 50 μl of the PPA (IS) solution, 100 μl of 0.2 M zinc sulfate, and 100 μl of 0.2 M barium hydroxide. The mixture was centrifuged at 6500 *g* for 20 min. The supernatant (deproteinized plasma; 200 μl) was mixed with 50 μl of 6 M hydrochloric acid, and the acidified plasma was hydrolyzed at 100 °C for 90 min. To the resulting solution, 2 ml of diethyl ether was added, and the resulting mixture was vortexed for *ca.* 2 min and centrifuged at 1000 *g* for 2 min. The organic layer (*ca.* 1.4 ml) was evaporated to dryness *in vacuo* at 15—20 °C and the residue was dissolved in 200 μl of acetonitrile. A 100-μl portion of the final solution was placed in a screw-capped 10-ml vial, to which were added *ca.* 20 mg of a mixture of potassium hydrogen carbonate and potassium sulfate (1 : 4, w/w) and 50 μl each of the Br-DMEQ and 18-crown-6 solutions. The vial was tightly closed and warmed at 50 °C for 30 min in the dark. After cooling, 20 μl of the resulting mixture was injected into the chromatograph. For the determination of free PAA, the same procedure was carried out except that hydrolysis was omitted.

The calibration graphs were prepared according to the standard procedure except that 50 μl of the PPA (IS) solution was replaced with the IS solution containing 50 pmol—100 nmol of PAA. The net peak height ratio of PAA was plotted against the concentration of PAA spiked.

Results and Discussion

HPLC and derivatization conditions were the same as described previously.[8]

Deproteinization

Plasma had to be deproteinized, otherwise the HPLC column packing was considerably

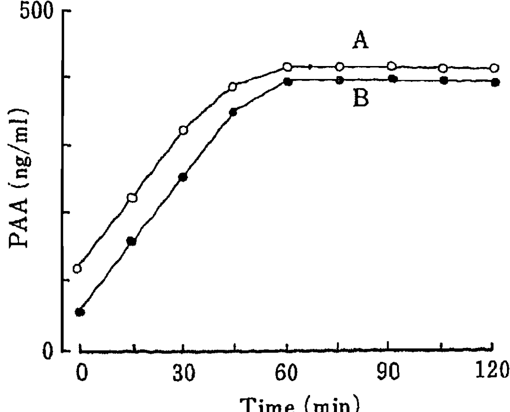


Fig. 1. Effect of the Reaction Time on the Hydrolysis of Conjugated PAA in (A) Human and (B) Control Rat Plasma

Portions (50 μl) of the plasma were treated according to the standard procedure.

damaged. The deproteinization was effectively done by adding zinc sulfate and barium hydroxide to plasma. When plasma was deproteinized with perchloric acid, an unknown, broad and large peak appeared at the retention time of 10—30 min on the chromatogram.

Hydrolysis

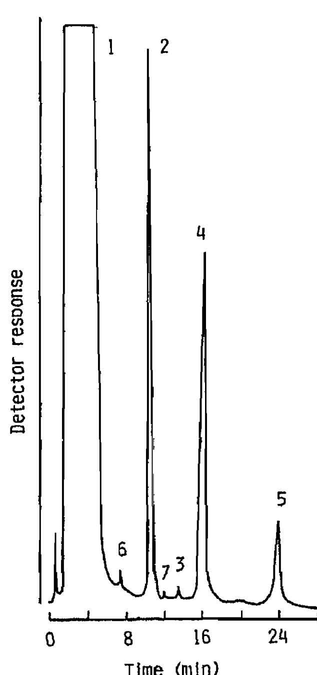
The optimal conditions for hydrolysis of conjugated PAA were examined by using pooled human and rat plasma. When the deproteinized plasma was acidified with an equal volume of 6 M hydrochloric acid, and hydrolyzed at 100 °C for 60—120 min, the conjugated PAA in human and rat plasma was almost completely hydrolyzed, as shown in Fig. 1. Thus, the acidified plasma was heated at 100 °C for 90 min in the procedure for the determination of total PAA.

Extraction

PAA was effectively extracted from the acidified plasma before and after hydrolysis with diethyl ether. A recovery test was performed by adding a known amount (50 pmol) of PAA to human plasma (50 μl). Recovery of PAA was $50.2 \pm 3.2\%$ (mean$\pm$S.D., $n=10$). Main loss in PAA occurred in the protein precipitation step. Less satisfactory recoveries were found with ethyl acetate, benzene and chloroform. Similar results were also obtained for rat plasma.

Chromatography

Figure 2 shows a typical chromatogram obtained with a standard mixture of PAA, *p*- and *m*-hydroxyphenylacetic acids and PPA.[8] The peaks for the acids (peaks 2—5) could be completely separated from the components of the reagent blank (Fig. 2, peaks 6 and 7) within 26 min. Figures 3A and B show typical chromatograms obtained with pooled human and rat plasma, respectively, before and after hydrolysis. The component of peak 2 was identified as the DMEQ derivative of PAA on the basis of the retention time and the fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra of the peak fraction by comparison with those in Fig. 2, and also by co-chromatography of the standard compound and plasma with aqueous 50—100% methanol as the mobile phase. On the other hand, no peaks for *p*- and *m*-hydroxyphenylacetic acids were observed in the chromatograms, because the acids occur in extremely small amounts in human plasma.[2,5,7] Some unidentified peaks (Fig. 3A and B, peaks 8—10) were observed on the chromatogram. The heights of these peaks increased in proportion to the plasma sample size. In addition, each eluate from peaks 8—10 exhibited fluorescence excitation and emission maxima around 370 and 455 nm, almost identical with those of peaks 2—5 (Fig. 2). These observations suggest that peaks 8—10 may be due to unknown endogeneous carboxylic acids in plasma. However, they did not interfere with the determination of PAA in plasma. No conversions of phenylacetaldehyde and



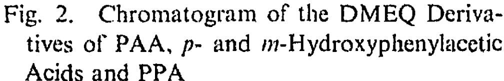


Fig. 2. Chromatogram of the DMEQ Derivatives of PAA, *p*- and *m*-Hydroxyphenylacetic Acids and PPA

A portion (50 μl) of a standard mixture of the acids (10 nmol each/ml) in water was treated according to the standard procedure. Peaks: 1, Br-DMEQ; 2, PAA; 3, *m*-hydroxyphenylacetic acid; 4, PPA; 5, *p*-hydroxyphenylacetic acid; 6 and 7, the reagent blank.

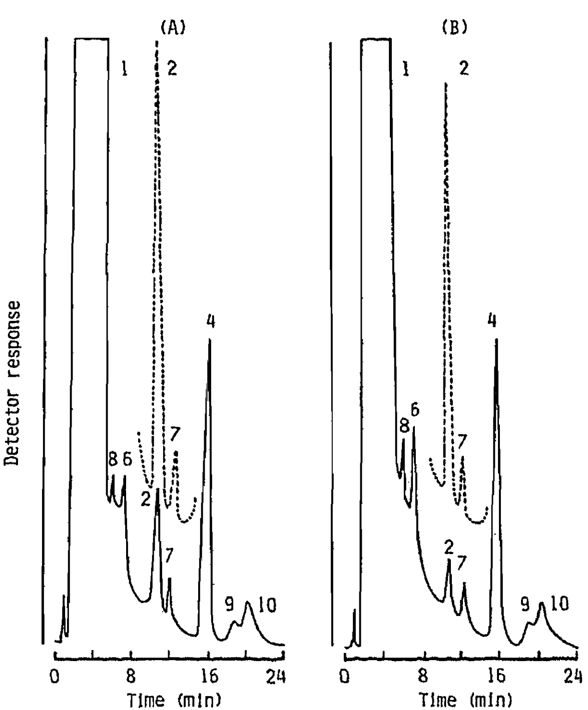


Fig. 3. Chromatograms Obtained with (A) Healthy Human and (B) Control Rat Plasma (——) before and (------) after Hydrolysis

Experimental details are described in the text. For peaks 1—7, see Fig. 2; peaks 8—10 are unidentified.

phenylpyruvic acid, which occur in biological fluids, to PAA during the procedure were observed even when they were present at unusually high concentrations in plasma (5.0 nmol/ml in plasma). Thus, further clean-up of the sample solution was not necessary.

Linearity, Detection Limit and Precision

A linear relationship was observed between the ratio of the peak height of PAA to that of PPA and the amounts of PAA (50 pmol—100 nmol) added to 50 μl of human plasma. The linear regression equation (the linear correlation coefficient in parenthesis) was $Y = 0.02053X + 0.0022$ ($r = 0.998$), where Y and X are the peak height ratio and the concentration (nmol/ml) of PAA, respectively.

The detection limit for PAA was 11 pmol/ml in plasma at a signal-to-noise ratio of 5. The sensitivity is much higher than that of the UV-HPLC method, and is comparable to those of the GC-MS methods.

The within-day precision was determined from repeated analyses ($n = 20$) of a normal human plasma containing 0.35 nmol/ml of free PAA and 2.93 nmol/ml of total PAA. The coefficients of variation were 3.9 and 3.6% for free and total PAA, respectively. The between-day precision was obtained by performing the analyses ($n = 3$ each day) using the calibration graph prepared on that day during ten days with plasma samples kept frozen at -40 °C. The coefficients of variation were 4.1 and 4.3% for free and total PAA, respectively.

PAA Concentration in Human Plasma

The levels of free, total and conjugated (calculated from differences) PAA in human plasma are given in Table I. The mean values were in agreement with those obtained by other

TABLE I. Plasma Concentrations of PAA (nmol/ml) in Normal Human

Age	Sex[a]	Free	Conjugate[b]	Total	Free/total (mol/mol)
59	M	1.81	3.09	4.90	0.37
37	M	0.49	1.77	2.26	0.22
35	M	0.44	1.45	1.89	0.23
31	M	0.34	1.49	1.83	0.18
28	M	0.45	1.42	1.87	0.24
27	M	0.54	1.63	2.17	0.25
27	M	0.67	3.03	3.70	0.22
24	M	0.57	1.71	2.28	0.25
24	M	0.52	1.55	2.07	0.25
23	M	0.37	1.72	2.09	0.18
Mean		0.62	1.89	2.58	0.24
S.D.		0.43	0.63	1.12	0.05
25	F	0.71	2.27	2.98	0.24
25	F	1.06	5.25	6.31	0.17
25	F	0.66	1.98	2.64	0.25
25	F	1.80	5.14	6.94	0.26
25	F	1.61	3.52	5.13	0.31
21	F	1.36	2.86	4.22	0.32
Mean		1.20	3.50	4.70	0.26
S.D.		0.47	1.41	1.75	0.05
Mean		0.84	2.50	3.34	0.25
S.D.		0.52	1.25	1.68	0.05

a) M, male; F, female. *b*) Conjugated PAA value is obtained by subtracting the free value from the total value.

TABLE II. Plasma Concentrations of PAA (nmol/ml) in Control Rats

	Free	Conjugate[a]	Total	Free/total (mol/mol)
1	0.23	2.13	2.36	0.10
2	0.51	3.54	4.05	0.13
3	0.32	2.83	3.15	0.10
4	0.33	2.00	2.33	0.14
5	0.47	4.06	4.53	0.10
6	0.31	1.81	2.12	0.15
7	0.48	3.12	3.60	0.13
8	0.32	2.56	2.88	0.11
9	0.31	2.46	2.77	0.12
10	0.19	1.31	1.50	0.13
Mean	0.35	2.58	2.93	0.12
S.D.	0.11	0.83	0.33	0.02

a) Conjugated PAA value is obtained by subtracting the free value from the total value.

workers.[1,2,4-6] The data indicated that plasma PAA concentration in females is significantly higher than that in males ($p<0.01$). The same observation was also reported by Davis *et al.*[2]

PAA Concentration in Rat Plasma

It is known that rats and mice, forced to swim in water, show a characteristic posture.

TABLE III. Plasma Concentration of Free and Total PAA (nmol/ml) in "Behavioral Despair" Rats

	Free	Conjugated[a]	Total	Free/total (mol/mol)
1	0.28	1.73	2.01	0.14
2	0.18	2.02	2.20	0.08
3	0.29	2.13	2.42	0.12
4	0.43	2.06	2.49	0.17
5	0.33	1.76	2.09	0.16
6	0.38	4.12	4.50	0.08
7	0.33	3.31	3.64	0.09
8	0.36	3.41	3.77	0.10
9	0.26	1.77	2.03	0.13
10	0.36	1.99	2.35	0.15
Mean	0.32	2.43	2.75	0.12
S.D.	0.07	0.85	0.88	0.03

a) Conjugated PAA value is obtained by subtracting the free value from the total value.

Porsolt *et al.* proposed this "behavioral despair" as an animal model of depression.[3] The concentrations of free and total PAA in plasma from control and "behavioral despair" rats determined by this method are shown in Table II. The levels of PAA in rat plasma were first determined by the present HPLC method. As shown in this Table, the mean values of free and total PAA in "behavioral despair" rat plasma were only slightly lower than those in control rat plasma, and a t test showed that the values were not significantly lower than those of the controls.

This study provides the first fluorimetric HPLC method for the assay of PAA in human and rat sera. The present method is highly sensitive; the sensitivity permits the assay of free and total PAA using a minute amount of plasma (50 μl). Namely, the method allows the determination of PAA in plasma of an experimental small animal. Although it remains to be seen whether the measurement of PAA provides significant information on brain function, this method should allow us to address the problem. The method is rapid and simple to perform, and could therefore be applied for routine use for the diagnosis and monitoring of depressive illness.

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Fluorometric Determination of Phenylpyruvic Acid with 1,4-Dimethyl-3-carbamoylpyridinium Chloride

AKIRA SANO,* MAKOTO OGAWA and SHOJI TAKITANI

Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

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A fluorometric method for the determination of phenylpyruvic acid (PPA) has been established. Benzaldehyde formed from PPA by alkaline decomposition is quantified by means of its fluorogenic reaction with 1,4-dimethyl-3-carbamoylpyridinium chloride. PPA can be determined in the range of 0.1—3 nmol/50 μl with the relative standard deviation of 4.0—4.1%. About 70% recovery was obtained when a urine sample (0.1 ml) added with 5 nmol of PPA was extracted and then determined by the proposed method. The method should be useful for the biochemical diagnosis of phenylketonuria.

Keywords——phenylpyruvic acid; benzaldehyde formation; 1,4-dimethyl-3-carbamoylpyridinium chloride; fluorometry; phenylketonuria

Phenylketonuria is a serious inherited disease involving a metabolic dysfunction. The determination of urinary phenylpyruvic acid (PPA) levels is considered to be applicable for the biochemical diagnosis of phenylketonuria, and several methods have been reported. However, at an early stage in the disease, the applicability of methods such as a qualitative test with test paper[1] and conventional spectrophotometric methods using 2,4-dinitrophenylhydrazine,[2] ferric chloride,[3] diazotized *p*-chloroaniline[4] and xanthene dye–aluminum (III) complex[5] is often limited by their low sensitivity. These methods also lack selectivity for PPA. Although gas chromatography[6] and high-performance liquid chromatography with fluorescence detection[7] are sensitive, the chromatographic steps are time-consuming.

PPA is known to give benzaldehyde when treated with alkali.[8] Further, we have recently proposed a sensitive and selective fluorometric method for determining aromatic aldehydes based on a fluorogenic reaction with 1,4-dimethyl-3-carbamoylpyridinium chloride (DCP-Cl) in the presence of alkali.[9] Fortunately, benzaldehydes having a hydroxy group, which would be expected to be formed from hydroxyphenylpyruvic acids, did not generate fluorescence in this method. It is shown here that PPA can be determined by reacting DCP-Cl with benzaldehyde produced by alkaline decomposition of PPA. A fluorometric method for determining PPA is proposed.

Experimental

Chemicals and Apparatus——DCP-Cl was synthesized by the published method.[9] A stock standard solution of PPA was prepared from PPA sodium salt (Sigma Chemical Co.) at 1 mM concentration; diluted solutions were also prepared by using water. Water used was purified on a Milli RO-Milli Q system (Millipore Ltd.). All other chemicals used were of analytical-reagent grade.

A Hitachi F-3000 fluorescence spectrometer equipped with a xenon lamp was used with a 10 × 10 mm quartz cell at room temperature; spectral bandwidths of 5 nm were used in both excitation and emission. All fluorescence excitation and emission spectra are uncorrected.

Recommended Procedure——Aqueous sample solution (50 μl) was taken in a 1.5 ml glass-stoppered test tube, and 50 μl of 5M aqueous sodium hydroxide solution containing 50 mM sodium pyrosulfite was added. The tube was heated

in a boiling water bath for 30 min, and then cooled in a water bath. Then 1 ml of 15 mM aqueous DCP-Cl solution was added. The mixture was incubated in a water bath at 37 °C for 50 min and then cooled to room temperature in a water bath. The fluorescence intensity was measured with excitation at 436 nm and emission at 506 nm.

Extraction of PPA from Urine——A urine sample (0.1 ml) was placed in a 10-ml glass-stoppered test tube, and 1 ml of water, 0.5 g of sodium chloride and 0.2 ml of 1 M hydrochloric acid were added. The mixture was extracted with 2-ml portions of diethyl ether (twice) by shaking for 5 min. To 3 ml of the combined ether extract, 0.5 ml of 2.5 M aqueous sodium hydroxide solution containing 25 mM sodium pyrosulfite was added. The mixture was shaken for 5 min. The organic layer was discarded, then a 0.1-ml aliquot of the aqueous layer was submitted to the fluorometric determination.

Results and Discussion

Fluorescence Excitation and Emission Spectra

The fluorescence excitation and emission spectra of the reaction mixture of PPA were identical with those obtained in the reaction of benzaldehyde with DCP-Cl. The wavelengths of maximal fluorescence excitation and emission of PPA were 436 nm and 506 nm, respectively.

Reaction Conditions

Analytical application of the decomposition reaction of PPA to benzaldehyde has not been reported. Thus the reaction conditions for benzaldehyde formation from PPA were first studied. Holcomb *et al.*[10] reported that conversion of *p*-hydroxyphenylpyruvic acid to *p*-hydroxybenzaldehyde was accomplished in 2—3 min at room temperature in 1 M sodium hydroxide. However, a higher temperature was necessary for the reaction of PPA in aqueous sodium hydroxide to form benzaldehyde. As shown in Fig. 1, constant fluorescence intensity was obtained on heating in a boiling water bath for 30—45 min, whereas longer times were necessary below 70 °C. Thus, reaction in a boiling water bath for 30 min was used.

The effect of sodium hydroxide concentration was then examined (Fig. 2). The highest fluorescence intensity was obtained when 2 M sodium hydroxide was used. However, 5 M solution was chosen, because it gave a better reproducibility and a lower blank value.

Reducing agents tended to decrease the blank fluorescence intensity. Sodium pyrosulfite was selected instead of sodium sulfite, which was used in the previous study,[9] because the former was more effective than the latter. Changes in the concentration of sodium pyrosulfite from 10—50 mM did not affect the fluorescence intensity of PPA; therefore, a concentration of 50 mM was chosen. Sodium hydroxide and sodium pyrosulfite could be used as a mixture.

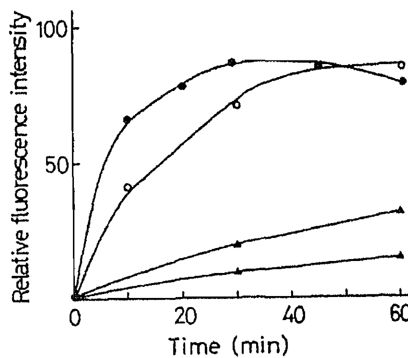

Fig. 1. Effect of Reaction Temperature and Time on the Formation of Benzaldehyde from PPA

PPA (2 nmol/50 μl) was treated by the recommended procedure except that various temperatures and times were used. Temperature: (△) 37 °C; (▲) 50 °C; (○) 70 °C; (●) 95 °C (boiling water bath).

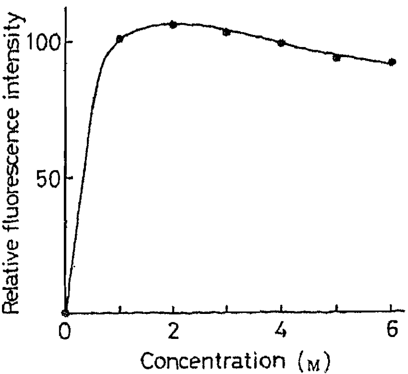

Fig. 2. Effect of Sodium Hydroxide Concentration on the Fluorogenic Reaction of PPA

PPA (2 nmol/50 μl) was treated by the recommended procedure except that various concentrations of aqueous sodium hydroxide were used.

Next, reaction conditions for the fluorogenic reaction of benzaldehyde liberated from PPA were examined. The fluorescence intensity was constant at DCP-Cl concentrations of 15—30 mM, and hence a 15 mM solution was used. The relationship between fluorescence intensity and the reaction time and temperature after addition of DCP-Cl was studied. The results obtained were similar to those described in the previous paper.[9] The best result was obtained when the reaction was done at 37 °C, and almost constant fluorescence intensity was obtained after 40—60 min. Thus conditions of 37 °C for 50 min were selected.

The fluorescence was stable for 40 min at room temperature.

Calibration Curve for Standard Solutions of PPA

A linear calibration curve was obtained over the concentration range of 0.1—3 nmol of PPA in 50 μl of sample. The relative standard deviations ($n=9$) were 4.1% at 0.3 nmol and 4.0% at 2 nmol.

Pyruvic acid, α-ketoglutaric acid, oxalacetic acid and *p*-hydroxyphenylpyruvic acid showed no fluorescence in the present method even at a concentration of 50 nmol/50 μl.

Recovery of PPA from Urine

The extraction procedure used was essentially the same as the method of Hirata and Ohkura[4] except that PPA was conveniently back-extracted from the ether extract into aqueous sodium hydroxide containing sodium pyrosulfite. The recovery test was performed by using normal urine because phenylketonuric urine could not be obtained. The recovery in this method was found to be $70.1 \pm 3.3\%$ (mean $\pm$ standard deviation) at the PPA concentration level of 5 nmol/0.1 ml urine ($n=4$). Fluorescence intensity resulting from blank urine was only about 1.5 times the reagent blank. When the first heating in a boiling water bath for 30 min was omitted in the fluorogenic reaction of urine extract containing PPA, the fluorescence intensity obtained was almost the same as that of the blank urine itself. Thus, in practice the blank value of the urine to be analyzed can be approximated by this fluorescence intensity. The lower limit of determination of PPA in urine was 1 nmol/0.1 ml (164 μg/100 ml). PPA can not be detected in normal urine by this method because of its concentration is very low (below about 0.2 mg in 24-h urine of adults and below about 3 μg in 24-h urine of newborn infants.[7]). However, the method should be easily applicable to PPA in urine from patients with phenylketonuria because urinary PPA levels in the disease are 24.0—132 mg/100 ml of urine.[11] The method can be used to distinguish clearly between normal and abnormal samples.

Phenylketonuria is also characterized by elevated levels of blood phenylalanine. It is well known that the urinary excretion of PPA is often delayed for several months as compared to the elevation of blood phenylalanine. Thus, current procedures used in mass screening for phenylketonuria usually consist of bioassay for phenylalanine in blood spotted on filter paper.[12] However, excessive amounts of phenylalanine are also found in blood from patients with hyperphenylalaninemia,[13] in which urinary excretion of PPA does not occur. Distinction between the diseases is necessary, because a phenylalanine-poor diet, which is used in phenylketonuria treatment, is not effective for hyperphenylalaninemia. The present method should be applicable for differential diagnosis.

In conclusion, the method described here seems to be useful for the biochemical diagnosis of phenylketonuria, because its sensitivity permits the determination of PPA in urine from patients even at considerably low levels.

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Fluorescent Koshland Reagent with Chelating Ability: Application to Selective Isolation of Tryptophan-Containing Peptide Fragments from Enzymatic Protein Digest

KEN'ICHI HAGIWARA,* HIROSHI NAKAMURA and TERUMI NAKAJIMA

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

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The fluorescent Koshland reagent, 2-carboxy-1-hydroxy-4-naphthylmethyldimethylsulfonium chloride (FKR), has three different functions: a) a phenolic hydroxy group *ortho* to carboxy group to chelate metal ions; b) a naphthyl moiety to produce fluorescence; and c) a Koshland reagent-type sulfonium chloride group to modify tryptophyl residues selectively in a peptide chain. Three procedures, methods I, II and III, using this reagent for the selective isolation of tryptophan-containing peptides from a protein digest were investigated. We selected lysozyme and carboxymethylated lysozyme as model proteins. In method I, lysozyme was labeled with FKR prior to the enzyme digestion, and the labeled protein was purified by gel permeation chromatography to remove the by-product formed from the reagent, then digested with protease. In method II, the protein (carboxymethylated lysozyme) was similarly labeled with the reagent, and the labeled protein was recovered as a precipitate by adding ethanol, leaving the excess reagent in the supernatant. The labeled protein was digested with protease. In method III, the protein was digested by protease before treatment with the reagent, and the by-product was removed by extraction with benzene. When the digests obtained by these procedures were applied to a Chelating Sepharose 6B column (Fe^{3+} or Al^{3+} form), FKR-labeled tryptophan-containing peptides were selectively adsorbed on the column owing to its chelating ability and could be eluted with 50 mM ethylenediaminetetraacetic acid. The final purification of these peptides was performed by reversed-phase high performance liquid chromatography on an octadecylsilane column in all cases.

Keywords——fluorescent Koshland reagent; tryptophan-containing peptide; Chelating Sepharose 6B chromatography; lysozyme; amino acid analysis

Recently, it has become possible to deduce the primary structures of protein rapidly from deoxyribonucleic acid (DNA) sequences. For the isolation of messenger ribonucleic acid (mRNA) or complementary deoxyribonucleic acid (cDNA) encoding a specific protein, chemically synthesized oligonucleotides are available as probes for hybridization techniques or as primers for reverse transcriptase on mRNA templates.[1-3] In such approaches, tryptophan and methionine are the most important amino acid residues because they have unique codons. That is, information about small portions of amino acid sequence containing tryptophan or methionine will be helpful to minimize the number of oligonucleotides to be synthesized and to identify the cloned cDNA. Therefore, a simple method for purification of tryptophan- or methionine-containing peptides is required.

In this paper, we report new methods for isolating tryptophan-containing peptides from protein digests. Our methods are based on modification of tryptophyl residues with the fluorescent Koshland reagent 2-carboxy-1-hydroxy-4-naphthylmethyldimethylsulfonium chloride (FKR), which we have previously synthesized[4] and have already applied to glucagon.[5] In addition to having similar reactivity to the conventional Koshland reagent,[6] FKR gives a fluorescent adduct with tryptophan (λ_{ex}255 /λ_{em}415 nm) owing to its naphthyl moiety, and excess reagent reacts with water to give a hydrolysis product (FKR-OH). This

reaction is completed within 5 min at room temperature. The most distinctive feature of FKR is that the reagent contains a salicylic acid structure which has chelating ability. Taking account of the solubility of proteins, removal of interfering FKR-OH and labeling yield, we devised three different methods and applied them to egg white lysozyme, which is known to have six tryptophyl residues per molecule.[7]

Experimental

Chemicals——Guanidine hydrochloride and 2-mercaptoethanol for biochemical use were purchased from Nakarai Chemicals, Kyoto, Japan. Iodoacetic acid was from Nakarai Chemicals, and was recrystallized from chloroform. Fluorescamine was from Roche, Basle, Switzerland. FKR was synthesized according to Hojo *et al.*[4] Sephadex G-15 and Chelating Sepharose 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Egg white lysozyme and porcine stomach pepsin were obtained from Sigma Chemicals Co., St. Louis, U.S.A. Trypsin was from Worthington Biochemical Corp., Freehold, U.S.A. Lysozyme was reduced and S-carboxymethylated according to the method of Crestfield *et al.*[8]

Apparatus——The spectrofluorometer used was a Hitachi type 650-10 (Hitachi Ltd., Tokyo, Japan). Reversed-phase high performance liquid chromatography (HPLC) was performed with a TRI ROTAR-VI chromatograph (JASCO, Tokyo, Japan) equipped with a Finepak SIL C_{18} column (10 μm, 4.6 i.d. × 250 mm) and a UVIDEC-100 ultraviolet absorption detector. The solvent system used was 5.7 mM HCl–acetonitrile (95:5, v/v) (A) and 5.7 mM HCl–acetonitrile (20:80, v/v) (B). Elution was carried out with a gradient from A to B as shown in the figures. Amino acid analysis was performed by the modified *o*-phthalaldehyde–sodium hypochlorite (OPA-NaOCl) method[9] using an IEX-215 column (4.6 i.d. × 50 mm, Toyo Soda, Tokyo, Japan).

Procedures for Isolating Tryptophan-Containing Peptides——Method I: Lysozyme (3 mg) was dissolved in 1 ml of 5 M guanidine hydrochloride solution and the pH was adjusted to 3 by adding glacial acetic acid. Solid FKR (10-fold molar excess over tryptophyl residues) was added to the solution with vigorous mixing at room temperature. After 10 min, the reaction mixture was applied on a Sephadex G-15 column (1.6 i.d. × 20 cm) equilibrated with 0.2 M acetic acid. The column was eluted with 0.2 M acetic acid and the eluate was monitored by measuring the native fluorescence due to the naphthyl moiety (Ex.255/Em.415 nm) and by fluorescamine assay (Ex.390/Em.475 nm).[10] The fractions containing labeled protein were adjusted to pH 2 with dilute HCl solution and incubated with 0.5 mg of pepsin at 37 °C for 20 h. After incubation, the digest was adjusted to pH 5 by adding dilute sodium hydroxide solution and applied to a Chelating Sepharose 6B column (Al^{3+} form, 1.0 i.d. × 10 cm equilibrated with 50 mM sodium acetate buffer (pH 5.0). The column was first washed with the same buffer to elute non-adsorbed components, and then with 50 mM ethylenediaminetetraacetic acid (EDTA) solution to recover adsorbed materials. Elution was monitored by measuring the naphthalene fluorescence and by fluorescamine assay. EDTA fractions containing peptides were lyophilized and resuspended in 5.7 mM HCl. The insoluble materials were removed by centrifugation and an aliquot of the supernatant was subjected to reversed-phase HPLC on a Finepak SIL C_{18} column. The purified peptides were hydrolyzed with a mixture of 5.7 M HCl and thioglycolic acid (100:4, v/v) at 110 °C for 20 h and amino acid compositions were determined by the OPA-NaOCl method.

Method II: Reduced carboxymethylated lysozyme (RCM-lysozyme) (1 mg) was dissolved in 150 μl of 5 M guanidine hydrochloride–0.2 M sodium acetate buffer (pH 4.0) and solid FKR (15-fold excess over tryptophyl residues) was added to the solution with continuous mixing. After incubation of the mixture at room temperature for 15 min, 10 ml of cold ethanol was added and the labeled protein was precipitated by centrifugation. The supernatant (which contained FKR-OH) was discarded and additional ethanol washing was performed twice. The resultant pellet was suspended in 500 μl of 5.7 mM HCl and digested with pepsin at 37 °C for 90 min. The digest was adjusted to pH 5, and successive chromatographies on the chelating column (Fe^{3+} form, 1.0 i.d. × 5.0 cm) and the octadecylsilane (ODS) column were performed as in the case of method I.

Method III: RCM-lysozyme (5 mg), suspended in 1 ml of dilute ammonia solution at pH 8.5, was digested with 50 μg of trypsin at 37 °C for 90 min. After digestion, the solution was adjusted to pH 3.0 with dilute HCl solution. Then, solid FKR (10-fold excess over tryptophyl residues) was added and mixed at room temperature for 10 min. The reaction mixture was shaken vigorously with 2 ml of benzene to extract FKR-OH. After centrifugation (800 × *g*, 3 min), the upper organic phase was carefully removed and additional benzene extraction was performed 4 times. Finally, the aqueous phase was adjusted to pH 5 and applied to the chelating column (Fe^{3+} form, 1.0 i.d. × 10 cm) in the same manner as in method I. Labeled peptides were purified through the use of the ODS column.

Results

Method I

Figure 1 shows the elution profile of labeled lysozyme on a Sephadex G-15 column. The

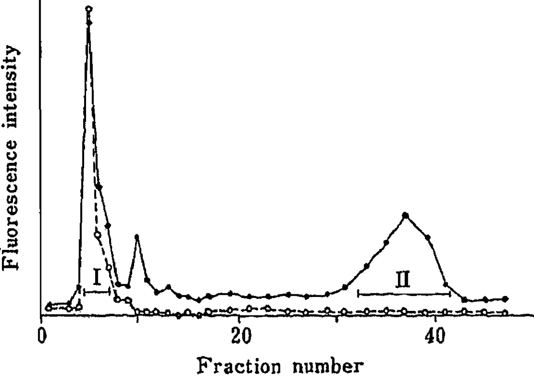

Fig. 1. Separation of Labeled Lysozyme from FKR-OH on a Sephadex G-15 Column

The reaction mixture was applied on a Sephadex G-15 column (1.6 i.d. ×20 cm) equilibrated with 0.2 M acetic acid. The column was eluted with the same solvent. ○—○, fluorescamine assay; ●—●, naphthalene fluorescence. I, labeled lysozyme; II, FKR-OH.

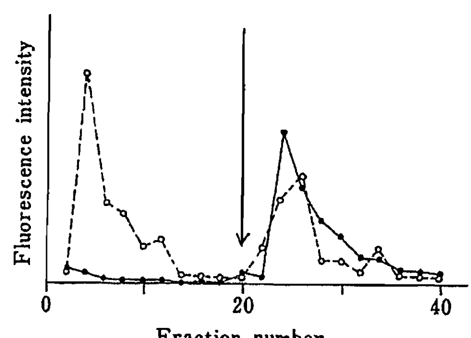

Fig. 2. Elution Profile of Labeled Lysozyme Fragments Resulting from Peptic Digestion on a Chelating Sepharose 6B Column

The column was washed with 50 mM sodium acetate buffer (pH 5.0), then elution was carried out with 50 mM EDTA solution (arrow). ○—○, fluorescamine assay; ●—●, naphthalene fluorescence.

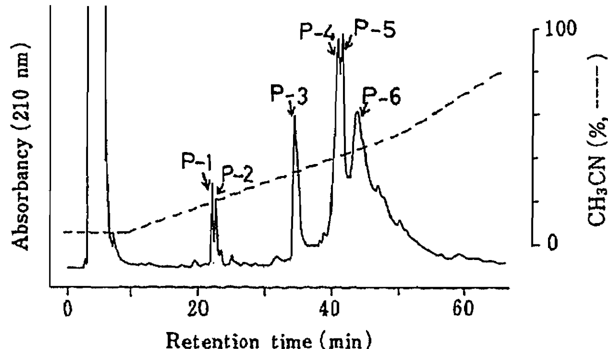

Fig. 3. Purification of Tryptophan-Containing Peptides by Reversed-Phase HPLC

EDTA fractions (obtained in Fig. 2) were subjected to HPLC on a Finepak Sil C_{18} column (10 μm, 4.6 i.d. ×250 mm). The column was eluted with acetonitrile gradient (----) at a flow rate of 1 ml/min. The column temperature was 40 °C. Elution was monitored by measuring the absorption at 210 nm.

Table I. Amino Acid Compositions of Acid Hydrolysates of Peptic Tryptophan-Containing Peptides

Peak	Amino acid composition	Assignment
P-1	Asx, 3.1 (3); Ser, 0.8 (1); Gly, 2.4 (3); Met, 0.9 (1); Leu, 1.2 (2); Tyr, 1.5 (2); His, 1.0 (1); Lys, 1.2 (1); Arg, 2.3 (2)	Met^{12}–Trp^{28}
P-2	Asx, 3.0 (3); Ser, 0.6 (1); Gly, 2.4 (3); Ala, 0.9 (1); Met, 0.9 (1); Leu, 1.3 (2); Tyr, 1.5 (2); His, 1.0 (1); Lys, 1.0 (1); Arg, 2.0 (2)	Ala^{11}–Trp^{28}
P-3	Glx, 1.1 (1); Gly, 2.0 (2); Ala, 1.8 (2); Val, 1.3 (1); Ile, 0.9 (1) Leu, 2.1 (2); Phe, 1.2 (1); Lys, 1.0 (1); Arg, 3.0 (3)	Lys^{1}—Cys^{6}—Ala^{10} Trp^{123}–Cys^{127}–Leu^{129}

Theoretical values are shown in parentheses.

labeled protein, separated from FKR-OH, was recovered in fraction I. Sephadex gel tended to adsorb FKR-OH, as indicated by its delayed elution. The labeled protein was digested with pepsin and applied to a chelating column (Fig. 2). The fractions from 24 to 26, which were positive for both naphthalene fluorescence and fluorescamine assay, were combined and lyophilized. The lyophilized sample was subjected to HPLC on an ODS column. As shown in Fig. 3, six separate fractions were obtained and their amino acid compositions were determined. From the amino acid compositions, P-1, P-2 and P-3 were assigned as the peptides listed in Table I. P-6 was considered to be the undigested labeled protein from the

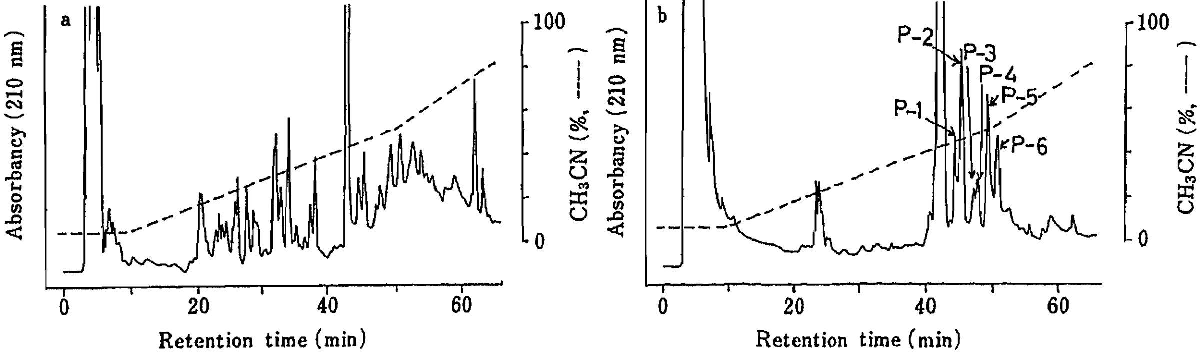

Fig. 4. Elution Profile of Labeled Lysozyme Fragments Resulting from Peptic Digestion on an ODS Column

The digest was subjected to HPLC before (a) or after (b) the chelating column chromatography. HPLC conditions were the same as in Fig. 3.

Table II. Amino Acid Compositions of Acid Hydrolysates of Peptide Tryptophan-Containing Peptides

Amino acid	P-1	P-2	P-3	P-4	P-5	P-6
Asx			1.3 (1)	1.7 (1)		1.3 (1)
Ser			0.9 (1)	1.0 (1)		1.0 (1)
Glx			1.0 (1)	0.9 (1)		
Gly	1.2 (1)			1.8 (1)		0.9 (1)
Ala		1.1 (1)			1.1 (1)	1.2 (1)
Val		1.0 (1)			1.0 (1)	0.9 (1)
Ile	0.9 (1)		0.8 (1)	1.7 (2)		
Leu	1.1 (1)			0.7 (1)		1.2 (1)
Tyr				0.7 (1)		0.7 (1)
Arg	2.2 (2)		0.8 (1)	1.2 (1)		
Cys	D[a] (1)	D (1)			D (1)	D (1)
Assignment	Trp^{123}–Leu^{129}	Trp^{28}–Ala^{31}	Gln^{57}–Trp$^{62\ or\ 63}$	Tyr^{53}–Trp$^{62\ or\ 63}$	Trp^{28}–Ala^{31}	Tyr^{23}–Ala^{31}

Theoretical values are shown in parentheses. a) Detected as carboxymethylated cysteine.

amino acid composition. P-4 and P-5 were considered to be a by-product of the reagent and FKR-OH, respectively, judging from their retention times. These were supposed to have been adsorbed at hydrophobic regions of the protein and not removed by gel permeation.

Method II

RCM-lysozyme was labeled with FKR, digested with pepsin and applied to the chelating column (data not shown) as described in Experimental. The number of peaks in the HPLC profile was greatly decreased after the pass through the chelating column (Fig. 4). Amino acid composition analysis indicated that the six peaks corresponded to tryptophan-containing peptides (Table II). A large peak at about 40 min after injection is residual FKR-OH as judged from the retention time. Indeed, no amino acid was detected in this peak fraction after acid hydrolysis.

Method III

As shown in Fig. 5, five fractions were obtained on the ODS column after the pass through the chelating column. From the amino acid composition, each fraction was assigned as a tryptophan-containing peptide, as shown in Table III. Although a small peak and a large peak appeared at about 40 min after injection, they are residual by-product of the reagent and

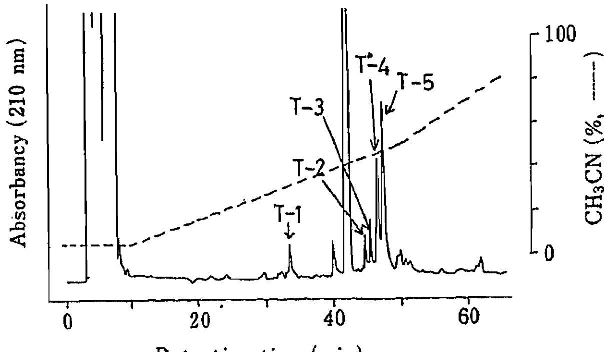

Fig. 5. Purification of Tryptophan-Containing Peptides by Reversed-Phase HPLC

The chromatographic conditions were the same as in Fig. 3.

TABLE III. Amino Acid Compositions of Acid Hydrolysates of Tryptic Tryptophan-Containing Peptides

Amino acid	T-1	T-2	T-3	T-4	T-5
Asx	2.3 (2)	1.6 (1)	3.4 (3)	1.0 (1)	1.0 (1)
Thr		0.7 (1)		0.8 (1)	
Ser			1.1 (1)		0.8 (1)
Glx		1.0 (1)		0.8 (1)	
Gly	1.1 (1)	1.2 (1)	2.5 (2)	1.4 (1)	2.1 (2)
Ala		1.2 (1)	1.8 (2)	1.2 (1)	2.4 (2)
Val		0.8 (1)	1.9 (2)	0.9 (1)	0.9 (1)
Met			0.8 (1)		
Ile			1.1 (1)	0.7 (1)	
Leu					0.9 (1)
Tyr					0.8 (1)
Lys					0.8 (1)
Arg	0.7 (1)		0.8 (1)	0.9 (1)	
Cys	D$^{a)}$ (1)				D (1)
Assignment	Trp^{62}–Arg^{68}	Gly^{117}–Trp^{123}	Ile^{98}–Arg^{112}	Gly^{117}–Arg^{125}	Gly^{22}–Lys^{33}

Theoretical values are shown in parentheses. *a*) Detected as carboxymethylated cysteine.

FKR-OH, as judged from the retention times. No amino acid was detected in these two peak fractions after acid hydrolysis.

Discussion

The results of this model study demonstrate that FKR is a useful tool for selective isolation of tryptophan-containing peptides. It should be noted that FKR not only reacts with tryptophyl residues but also introduces chelating ability into the labeled peptides. For the separation of tryptophan-containing peptides, several methods have been reported. Sasagawa *et al.*[11] and Tanaka *et al.*[12] took advantage of hydrophobic modification and changes of chromatographic mobility on a reversed-phase column. These are simple methods, but the possibility remains that modified tryptophan-containing peptides may be coeluted with other residual peptides on reversed-phase HPLC. An affinity Sepharose column coated with anti-dinitrophenyl antibodies was proposed by Wilchek and Miron.[13] Rubinstein *et al.*[14] used polymeric reagents as covalent chromatography resins. These two methods are comparable to ours in that only tryptophan-containing peptides are picked up by adsorption on the columns, but our methods are preferable because of the simplicity of preparation of the column.

Initially, we applied method I to lysozyme. The removal of FKR-OH was achieved most

easily by gel permeation chromatography without serious loss of labeled protein, though a small amount of FKR-OH which might have been adsorbed at hydrophobic regions of the protein remained. Thus, method I is a recommended standard procedure. However, method I is inappropriate for insoluble proteins which can not be subjected to gel permeation chromatography without a solubilizing reagent (for example, many reduced carboxymethylated proteins or membrane proteins). By method I, we succeeded in recovering only two tryptophyl residues (*i.e.*, Trp^{28} and Trp^{123}) among the six tryptophyl residues in lysozyme. This low recovery can be explained mainly by incomplete digestion of the labeled protein owing to its rigid tertiary structure. Steric hindrance to labeling should be small, since all tryptophyl residues of lysozyme are exposed to solvents in the presence of 5 M guanidine hydrochloride.[15] For the purpose of optimizing the extent of digestion, reduction of disulfide bonds followed by carboxymethylation was performed. This treatment made the protein quite insoluble in water and it was found that method I was unsuitable. Therefore, two additional methods (*i.e.*, methods II and III) were developed. We obtained four of the six tryptophyl residues in lysozyme by method II (Table II). Method II is unsuitable for proteins which are rather soluble in ethanol (for example, bovine serum albumin). By using method III, we purified all the tryptophyl residues in lysozyme (Table III). It is difficult to get a high labeling yield by using method I or II if tryptophyl residues are sterically hindered within a protein molecule. Even for such "concealed" residues, a higher labeling yield is expected by method III if steric hindrance is eliminated thoroughly by digestion. The defect in method III is its low efficiency for removal of FKR-OH. We attempted to remove FKR-OH by benzene extraction, but the extraction efficiency was not adequate. The conventional Koshland reagent is known to give more than two products with tryptophan.[6] In method II, FKR also gave two products which are distinguishable in retention time from a single peptide (Trp^{28}–Ala^{31}) derived from lysozyme. In the present study, this was the only case in which more than one product was obtained from one peptide.

We examined two kinds of chelating resins, Chelating Sepharose 6B and Chelex-100 (Bio-Rad). The recovery of peptides from the former resin was better than that from the latter. The peptides were eluted with tailing on a Chelex-100 column, presumably because of hydrophobic interaction. For this reason, Chelating Sepharose 6B resin was chosen for our study. Al^{3+} was used in method I and Fe^{3+} in methods II and III. Fe^{3+} showed better reproducibility than Al^{3+} so far as we examined.

The methods described here have different properties from one another. The most suitable one can be selected depending on the given protein, the purposes of research and so on. It is true that reversed-phase HPLC is widely used for purification of peptides, but adequate resolution is not always obtained, especially for complex mixtures. Our methods, by which tryptophan-containing peptides can be selectively isolated, facilitate purification by HPLC and should be advantageous for sequence analysis of proteins.

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Synthesis of Calf Thymosin β_4 Fragment 16—38 and Its Effect on the Impaired Blastogenic Response of T-Lymphocytes of a Uremic Patient with Pneumonia[1]

TAKASHI ABIKO* and HIROSHI SEKINO

Kidney Research Laboratory, Kojinkai, Higashishichiban-cho 84, Sendai 980, Japan

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Thymosin β_4 fragment 16—38, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–OH, was synthesized by a solution method, by assembling five peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The synthetic tricosapeptide was tested for effect on the impaired blastogenic response of phytohemagglutinin-stimulated T-lymphocytes of a uremic patient with pneumonia. The synthetic tricosapeptide was found to have restoring activity, which was approximately equal in potency to that of our synthetic deacetyl-thymosin β_4.

Keywords——thymosin β_4 fragment 16—38; trifluoromethanesulfonic acid deprotection; uremic patient; T-lymphocyte impaired blastogenic response; fluorometric blast-formation test

Thymosin β_4 exhibits several biological activities that are important for maturation and functioning of the immune system in man and animals[2,3] and consists of 43 amino acid residues. The amino acid sequences of polypeptides β_8 and β_9, which were isolated from calf thymus by Hannappel *et al.*, were found to be homologous to thymosin β_4.[4] As shown in Fig. 1, 31 out of the 39 amino acid residues present in thymosin β_8 are identical to the corresponding residues in thymosin β_4. Thymosin β_9 is identical to β_8 except for the presence of an additional dipeptide, –Ala–Lys–OH, at the C-terminus.

In the previous papers,[5-7] we reported syntheses of thymosin β_8, thymosin β_9 and deacetyl-thymosin β_4, and showed that these synthetic thymus peptides could have restoring

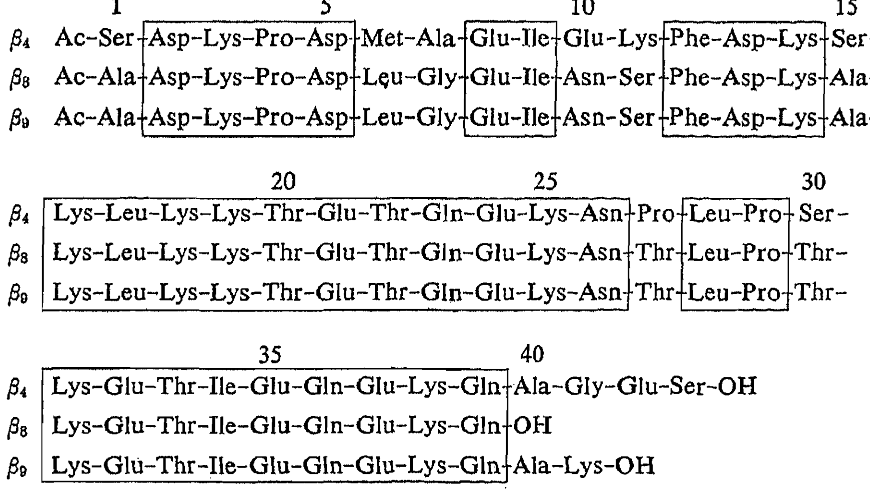

Fig. 1. Comparison of Amino Acid Sequences of Thymosins β_4, β_8 and β_9

Identical sequences are enclosed in Boxes.

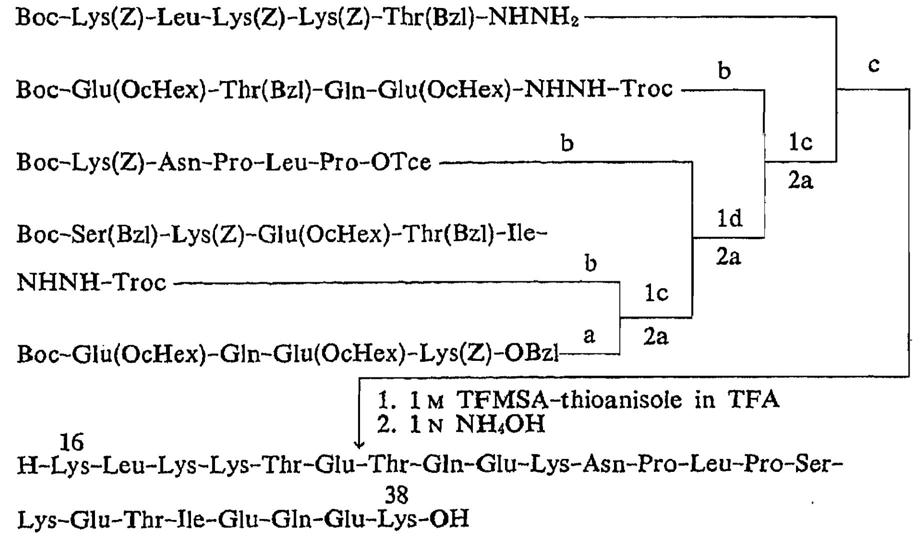

Fig. 2. Synthetic Route to Thymosin β_4 Fragment 16—38

a, TFA–anisole; b, Zn–AcOH; c, azide; d, HOSu–WSCI.

effects on the impaired cell-mediated immunological functions. We also reported that two fragments[8] of thymosin β_4, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (16—26) and H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–OH (31—39), and a fragment[9] of thymosin β_9, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–OH (16—25), showed *in vitro* immunological activities on low E-rosette-forming cells of patients with cell-mediated immunodeficiencies.

We are interested in the peptide fragment corresponding to amino acids 16 to 38 of thymosin β_4, which contains two immunologically active sites, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–OH (16—26) and H–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–OH (31—38) within its molecule. We describe here the synthesis of thymosin β_4 fragment 16—38, H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–OH. Further, we have compared *in vitro* effects of the synthetic tricosapeptide and synthetic deacetyl-thymosin β_4 on the impaired response to PHA stimulation of T-lymphocytes from a uremic patient with recurrent pneumonia.

The methods we employed here are essentially the same as those employed for our previous synthesis of deacetyl-thymosin β_4.[7] However, several improvements have been made in the present synthesis, as illustrated in Fig. 2, as compared with our previous synthesis of thymosins[5-7] and their fragments.[8,9] The thioanisole-mediated TFMSA deprotecting procedure[10,11] was employed. Besides Lys(Z), Thr(Bzl) and Ser(Bzl), a new amino acid derivative bearing a protecting group removable by 1 M TFMSA–thioanisole in TFA was employed, *i.e.*, Glu(OcHex).[12] Glu(OcHex) was employed to suppress base-catalyzed pyrrolidone formation.[13] These protecting groups survive mostly intact under careful TFA treatment for removal of the Boc group, employed as a temporary α-amino protecting group.

As shown in Fig. 2, five fragments were selected as building blocks to construct the tricosapeptide fragment 16—38 of thymosin β_4. Hydrazides containing Glu(OcHex) were synthesized with the aid of substituted hydrazine, Troc–$NHNH_2$,[14] the protecting groups of which can be removed by Zn[15] in AcOH without affecting side chain protecting groups such as Boc, Z, Bzl and OcHex. Throughout the syntheses of these intermediates and fragments, the purity of every fragment and intermediate was checked by TLC, elemental analysis and amino acid analysis. Boc. groups of intermediates were removed by treatment with TFA–

anisole prior to the next coupling reaction.

First, the C-terminal fragment, Boc–(35—38)–OBzl, was prepared stepwise starting from Boc–Lys(Z)–OBzl by the HOBT–WSCI procedure[16] except for the introduction of a Gln residue, which was introduced by the NP active ester procedure.[17] Next, the two fragments containing Glu(OcHex), Boc–(30—34)–NHNH–Troc and Boc–(21—24)–NHNH–Troc, were prepared stepwise by the HOBT–WSCI procedure[16] except for the introduction of a Gln residue, which was introduced by the NP active ester procedure.[17] The two fragments thus obtained were treated with Zn[15] in AcOH to remove Troc groups, and the zinc acetate was removed by treatment with EDTA to give the required hydrazides in analytical pure form. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired products. Thus these fragments were prepared without exposing the corresponding ester to hydrazide. Then, Boc–(25—29)–OTce, was prepared stepwise starting from Boc–Pro–OTce by the HOBT–WSCI procedure[16] except for the introduction of an Asn residue, which was introduced by the NP active ester procedure.[17] During the courses of NP steps, HOBT was used as a coupling reaction accelerator.[16] The resulting pentapeptide ester was treated with Zn[18] in AcOH–H_2O (4 : 1) to remove the Tce, and the zinc acetate was removed by treatment with EDTA to give Boc–(25—29)–OH in analytically pure form.

Finally, the N-terminal fragment, Boc–(16—20)–OBzl, was prepared stepwise by the HOBT–WSCI procedure.[16] The pentapeptide ester thus obtained was converted to the corresponding hydrazide, Boc–(16—20)–$NHNH_2$, in the usual manner. The hydrazine test on the thin-layer chromatograms and elemental analysis data of the peptide fragment were consistent with homogeneity of the desired product.

The five fragments thus obtained were then assembled successively by the azide procedure[18] and the HOSu–WSCI procedure[19] according to the routes illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 1.6 to 3 eq as the chain elongation proceeded. The solubility of protected intermediates in DMF decreased remarkably with chain elongation. Consequently, mixtures of DMF–DMSO had to be employed for the subsequent condensation reactions.

The first fragment condensation product, Boc–(30—38)–OBzl, was purified by gel-filtration on Sephadex LH-20 using DMF containing 3% H_2O as the eluent and the other three intermediates were purified by repeated precipitation from DMF or DMSO with MeOH. Throughout this synthesis, Ile was taken as a diagnostic amino acid. Each intermediate was subjected to acid hydrolysis and the recovery of Ile was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each condensation. The homogeneity of every intermediate was further ascertained by elemental analysis and TLC. Moreover, we attempted to take advantage of the well-known fact that D-allo-Ile or D-allo-Thr is easily separable from the parent amino acid, Ile or Thr, by using an amino acid analyzer to detect the degree of racemization during the coupling reactions.[20] For this purpose, we chose the two synthetic fragments, Boc–(30—34)–$NHNH_2$ and Boc–(16—20)–$NHNH_2$, bearing Ile and Thr in the C-terminus, respectively. Fortunately, after coupling of these fragments, we could not detect any D-allo-Ile or D-allo-Thr in the resulting peptides.

In the final step of the synthesis, the protected tricosapeptide benzyl ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of $(Me)_2Se$ in an ice-bath for 100 min to remove all the protecting groups employed. $(Me)_2Se$ was used as an accelerator for the cleavage of protecting groups.[21] The deprotected peptide was next precipitated with dry ether, converted to the corresponding acetate with Amberlite IR-400 (acetate form) and then treated with 1 N NH_4OH to reverse a possible N→O shift at the Ser and Thr residues.[22] The crude peptide was purified by gel-filtration on Sephadex G-25 and then ion-exchange column chromatography on a CM-Sephadex C-25 column with a linear gradient of pH 6.51

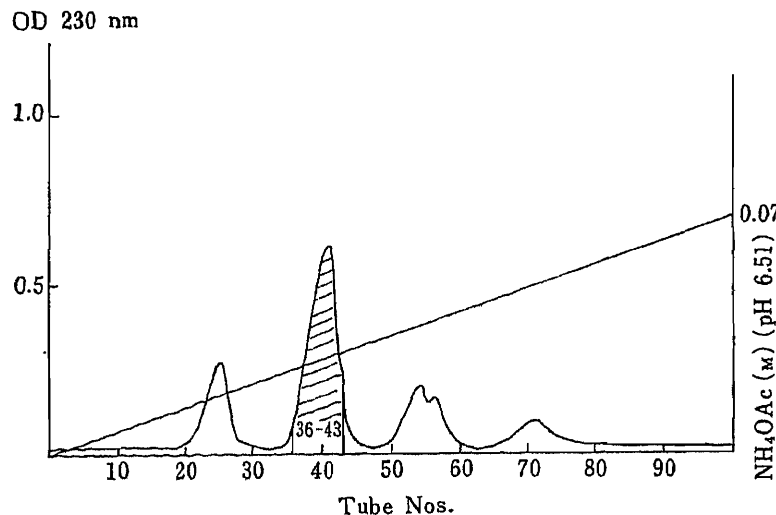

Fig. 3. Purification of Synthetic Thymosin β_4 Fragment 16—38 on a Column of CM-Sephadex C-25

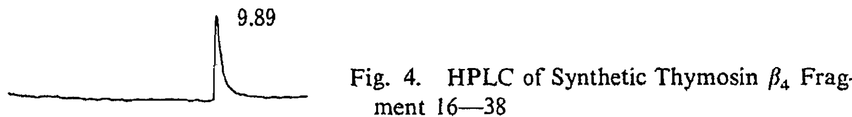

Fig. 4. HPLC of Synthetic Thymosin β_4 Fragment 16—38

TABLE I. Effects of the Synthetic Deacetyl-Thymosin β_4 and Its Two Fragments on the Impaired Blastogenic Response of T-Lymphocyte of a Uremic Patient with Pneumonia

Peptides	Dose (M)	$SI^{a,b)}$	Relative potency
—[c)]	—	269.3±58.2	
—[d)]	—	113.7±60.3[g)]	
Deacetyl-thymosin β_4[d,e)]	4.9×10^{-8}	174.3±55.6[h)]	
	4.9×10^{-7}	218.6±59.4[h)]	1.00
Fragment 16—38[d,e)]	2.8×10^{-8}	180.1±56.3[h)]	
	2.8×10^{-7}	220.4±51.9[h)]	0.96
Fragment 12—14[d,e,f)]	4.1×10^{-7}	116.1±61.4	
	4.1×10^{-5}	110.2±54.7	—

a) Each value represents the mean±S.D. of triplicate measurements. *b*) *SI* (stimulation index) was calculated according to the following formula $SI=\frac{I_2-I_0}{I_1-I_0}\times100$, where I_2=mean fluorescence intensity of PHA-P-activated lymphocytes, I_1=fluorescence intensity of PHA-P-nonactivated lymphocytes and I_0= fluorescence intensity of ethydium bromide. *c*) Normal peripheral lymphocytes. *d*) Patient's peripheral lymphocytes. *e*) Incubation was carried out for 12 h at 37 °C. *f*) This peptide[8)] had no effect on the impaired blastogenic response of PHA-stimulated T-lymphocytes at the concentration of 4.1×10^{-5} M. *g*) $p<0.05$, when compared to normal persons by using Student's *t*-test. *h*) $p<0.01$, when compared to the uremic patient by using Student's *t*-test.

ammonium acetate buffer (0→0.07 M), followed by preparative TLC. The tricosapeptide thus obtained was homogeneous on TLC in two different solvent systems and paper electrophoresis (pH 3.1 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 4). Its purity was further confirmed by amino acid analysis after acid hydrolysis and

enzymatic digestion. Despite the presence of the Pro residues,[23] complete digestion of this synthetic peptide with commercial aminopeptidase (AP-M)[24] was achieved and the presence of Asn and Gln residues in the product was thus confirmed.

The immunological effects of the synthetic deacetyl-thymosin β_4[7] and the synthetic thymosin β_4 fragment 16—38, were examined by means of the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[25] In contrast to normal persons, the blastogenesis of the T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in a uremic patient with pneumonia. The *in vitro* effects of the synthetic peptides on the impaired PHA response of T-lymphocytes from a uremic patient with pneumonia are shown in Table I.

Comparison of the SI values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity by PHA stimulation shows that, in the case of the patient investigated, the synthetic tricosapeptide restored the blastogenesis to nearly normal values at a dose of 2.8×10^{-7} M. The mitogenic activity of the synthetic tricosapeptide was approximately equal to that of the synthetic deacetyl-thymosin β_4 at a concentration of 2.8×10^{-7} M. However, the synthetic thymosin β_4 fragment 12—14 did not restore the impaired mitogenic response of the T-lymphocytes at a high concentration of 4.1×10^{-5} M. These result suggest that a portion of the amino acid sequence of thymosin β_4, –Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys– (16—38), is an important moiety in thymosin β_4 for the restorative activity on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients with recurrent infectious disease such as pneumonia.

Experimental

Azides were prepared according to Honzl and Rudinger[18] with isoamyl nitrite. Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates were determined with a Hitachi 835-50 type amino acid analyzer. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—40 °C. Boc groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf^1, the Partridge system[26]; Rf^2, BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] The final product corresponding to the thymosin β_4 fragment 16—38 was chromatographed on a cellulose plate (Merck) at room temperature. Rf^3 value refers to the Partridge system[26] and Rf^4 to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] Troc–$NHNH_2$ was purchased from the Japan Immunoresearch Laboratories Co., Ltd., Japan. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Aminopeptidase (3501, Aminopeptidase 210520) was purchased from the Peptide Institute Inc., Minoh, Osaka 562, Japan. Venous blood was obtained from a uremic patient suffering from pneumonia. Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with a UVLOG-FLOUSPEC 11 A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a μBondapak C_{18} column.

Boc–Glu(OcHex)–Lys(Z)–OBzl (I)——Boc–Lys(Z)–OBzl (2.4 g) was treated with TFA–anisole (12 ml–2.2 ml) in an ice-bath for 40 min, and TFA was then removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF (20 ml) containing NMM (0.6 ml). To this ice-chilled solution, Boc–Glu(OcHex)–OH (1.8 g), HOBT (743 mg) and WSCI (1.1 g) were successively added. After having been stirred at 0 °C for 8 h, the mixture was extracted with EtOAc, and the extract was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$, and then concentrated *in vacuo*. The residue was reprecipitated from EtOAc with *n*-hexane. Yield 2.6 g (74%), mp 92—96 °C, $[\alpha]_D^{21}$ $-13.6°$ (c=1.0, DMF), Rf^1 0.51, Rf^2 0.58, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{51}N_3O_9 \cdot 2H_2O$: C, 61.91; H, 7.72; N, 5.85. Found: C, 61.99; H, 7.83; N, 5.72.

Boc–Gln–Glu(OcHex)–Lys(Z)–OBzl (II)——I (2.3 g) was treated with TFA–anisole (11 ml–2.1 ml) as usual and the resulting powder was dissolved in DMF (14 ml) together with NMM (0.37 ml). HOBT (500 mg) and Boc–Gln–ONp (1.6 g) was added and the solution was stirred at room temperature for 7 h. The reaction mixture was diluted with 1 N NH_4OH (4 ml) with stirring to saponify the unchanged *p*-nitrophenyl ester. After 1 h, the mixture was poured into ice-chilled 1 N NH_4OH with vigorous stirring. The precipitate formed was washed successively with 1 N NH_4OH,

H_2O, 5% citric acid and H_2O. The product was reprecipitated from DMF with H_2O. Yield 2.1 g (78%), mp 141—147 °C, $[\alpha]_D^{21}$ −21.4° (c=1.0, DMF), Rf^1 0.50, Rf^2 0.51, single ninhydrin-positive spot. *Anal.* Calcd for $C_{42}H_{59}N_5O_{11}\cdot 1^1/_2H_2O$: C, 60.27; H, 7.47; N, 8.37. Found: C, 60.13; H, 7.59; N, 8.52.

Boc–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–OBzl (III)——This compound was prepared essentially in the same manner as described for the preparation of I by using II (1.6 g), Boc–Glu(OcHex)–OH (723 mg), HOBT (297 mg) and WSCI (422 mg). The product was recrystallized from MeOH with ether. Yield 1.5 g (80%), mp 142—149 °C, $[\alpha]_D^{21}$ −5.6° (c=1.0, DMF), Rf^1 0.60, Rf^2 0.58, single ninhydrin-positive spot. *Anal.* Calcd for $C_{53}H_{76}N_6O_{14}$: C, 62.34; H, 7.50; N, 8.23. Found: C, 62.06; H, 7.68; N, 7.98.

Boc–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–NHNH–Troc (IV)——This compound was prepared essentially in the same manner as described for the preparation of I by using Boc–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–NHNH–Troc[28] (1.1 g), Boc–Ser(Bzl)–OH (325 mg), HOBT (149 mg) and WSCI (211 mg). The product was reprecipitated from MeOH with ether. Yield 849 mg (66%), mp 136—142 °C, $[\alpha]_D^{21}$ −6.8° (c=1.0, DMF), Rf^1 0.66, Rf^2 0.71, single ninhydrin-positive spot. *Anal.* Calcd for $C_{60}H_{83}Cl_3N_8O_{15}\cdot H_2O$: C, 57.62; H, 6.85; N, 8.96. Found: C, 57.66; H, 6.93; N, 8.72.

Boc–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–NHNH$_2$ (V)——IV (702 mg) in a mixture of AcOH (8 ml) and DMF (8 ml) was treated with Zn dust (780 mg) at 4 °C for 2 h and then at room temperature for 10 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA to form a powder, which was washed with 5% $NaHCO_3$ and H_2O. The product was reprecipitated from DMF with H_2O. Yield 528 mg (87%), mp 179—187 °C, $[\alpha]_D^{21}$ −15.4° (c=1.0, DMF), Rf^1 0.40, Rf^2 0.48, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{57}H_{82}N_8O_{13}\cdot 3H_2O$: C, 59.98; H, 7.77; N, 9.82. Found: C, 59.75; H, 7.83; N, 9.56.

Boc–Leu–Pro–OTce (VI)——This compound was prepared essentially in the same manner as described for the preparation of I by using Boc–Pro–OTce (1.2 g), Boc–Leu–OH (914 mg), HOBT (495 mg) and WSCI (703 mg). The product was reprecipitated from EtOAc with petroleum ether. Yield 1.5 g (94%) (oily material), $[\alpha]_D^{21}$ −14.5° (c=1.0 DMF), Rf^1 0.66, Rf^2 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{18}H_{29}Cl_3N_2O_5$: C, 47.02; H, 6.36; N, 6.09. Found: C, 46.87; H, 6.48; N, 5.94.

Boc–Pro–Leu–Pro–OTce (VII)——This compound was prepared from VII (1.2 g), Boc–Pro–OH (592 mg), HOBT (372 mg) and WSCI (527 mg) essentially as described for the preparation of I. The product was precipitated from EtOAc with petróleum ether. Yield 1.1 g (73%) (oily material), $[\alpha]_D^{21}$ −26.9° (c=1.0, DMF), Rf^1 0.82, Rf^2 0.88, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{36}Cl_3N_3O_6$: C, 49.60; H, 6.52; N, 7.55. Found: C, 49.42; H, 6.59; N, 7.38.

Boc–Asn–Pro–Leu–Pro–OTce (VIII)——VII (1 g) was treated with TFA–anisole (8 ml–1.6 ml) as described above and the resulting oily material was dissolved in DMF (10 ml) together with NMM (0.2 ml), HOBT (270 mg) and Boc–Asn–ONp (729 mg). The solution was stirred at room temperature for 6 h. The reaction mixture was diluted with 1 N NH_4OH (3 ml) with stirring to saponify unchanged *p*-nitrophenyl ester. After 1 h, the mixture was extracted with EtOAc, and the extract was washed successively with 1 N NH_4OH, H_2O, 5% citric acid and H_2O, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was precipitated from EtOAc with *n*-hexane. Yield 923 mg (77%), mp 112—116 °C, $[\alpha]_D^{21}$ −21.4° (c=1.0, DMF), Rf^1 0.65, Rf^2 0.67, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{42}Cl_3N_5O_8\cdot H_2O$: C, 47.07; H, 6.44; N, 10.16. Found: C, 47.16; H, 6.72; N, 10.05.

Boc–Lys(Z)–Asn–Pro–Leu–Pro–OTce (IX)——This compound was prepared essentially in the same manner as described for the preparation of I by using VIII (671 mg), Boc–Lys(Z)–OH·DCHA (618 mg), HOBT (149 mg) and WSCI (211 mg). The product was reprecipitated from EtOAc with ether. Yield 624 mg (67%), mp 106—109 °C, $[\alpha]_D^{21}$ −11.2° (c=1.0, DMF), Rf^1 0.70, Rf^2 0.72, single ninhydrin-positive spot. *Anal.* Calcd for $C_{41}H_{60}Cl_3N_7O_{11}$: C, 52.76; H, 6.48; N, 10.51. Found: C, 52.47; H, 6.72; N, 10.60.

Boc–Lys(Z)–Asn–Pro–Leu–Pro–OH (X)——IX (549 mg) in a mixture of AcOH (4 ml) and MeOH (4 ml) was treated with Zn dust (165 mg) at room temperature for 6 h. Fresh Zn dust (80 mg) was added and the solution, after being stirred for an additional 3 h, was filtered. The residue was dissolved in EtOAc. The organic phase was successively washed with 3% EDTA, 5% citric acid and H_2O, and concentrated *in vacuo*. Treatment of the residue with ether afforded a powder. Yield 427 mg (92%), mp 95—101 °C, $[\alpha]_D^{21}$ −28.1° (c=1.0, DMF), Rf^1 0.34, Rf^2 0.32, single ninhydrin-positive spot. *Anal.* Calcd for $C_{29}H_{59}N_7O_{10}\cdot H_2O$: C, 58.27; H, 7.65; N, 12.20. Found: C, 58.19; H, 7.75; N, 12.10.

Boc–Gln–Glu(OcHex)–NHNH–Troc (XI)——This compound was prepared from Boc–Glu(OcHex)–NHNH–Troc (2.6 g), Boc–Gln–ONp (2.2 g) and HOBT (690 mg) essentially as described for the preparation of VIII. The product was reprecipitated from EtOAc with *n*-hexane. Yield 3.0 g (94%), mp 130—137 °C, $[\alpha]_D^{21}$ 15.3° (c=1.0, DMF), Rf^1 0.48, Rf^2 0.44, single ninhydrin-positive spot. *Anal.* Calcd for $C_{24}H_{38}Cl_3N_5O_9\cdot H_2O$: C, 43.35; H, 6.06; N, 10.53. Found: C, 43.21; H, 6.27; N, 10.28.

Boc–Thr(Bzl)–Gln–Glu(OcHex)–NHNH–Troc (XII)——This compound was prepared from XI (2.2 g), Boc–Thr(Bzl)–OH (1.1 g), HOBT (495 mg) and WSCI (703 mg) essentially as described for the preparation of I. The product was precipitated from MeOH with ether. Yield 1.8 g (62%), mp 142—150 °C, $[\alpha]_D^{21}$ +4.9° (c=1.0, DMF), Rf^1 0.62, Rf^2 0.70, single ninhydrin-positive spot. *Anal.* Calcd for $C_{35}H_{51}Cl_3N_6O_{11}$: C, 50.16; H, 6.13; N, 10.03. Found: C, 50.49; H, 6.40; N, 10.18.

Boc–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–NHNH–Troc (XIII)——XII (1.4 g) was treated with TFA–anisole (10 ml–2 ml) as described above, and the resulting powder was dissolved in DMF (10 ml) containing NMM (0.19 ml). To this ice-chilled solution, Boc–Glu(OcHex)–OH (602 mg), HOBT (248 mg) and WSCI (352 mg) were successively added. After having been stirred at 0 °C for 10 h, the mixture was poured into an ice-chilled 5% citric acid, and the precipitate thereby formed was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was recrystallized from hot EtOAc. Yield 1.5 g (83%), mp 151—160 °C, $[\alpha]_D^{21}$ −13.2° (c=1.0, DMF), Rf^1 0.59, Rf^2 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{46}H_{68}Cl_3N_7O_{14}\cdot 2H_2O$: C, 50.90; H, 6.69; N, 9.03. Found: C, 50.82; H, 6.83; N, 9.14.

Boc–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–$NHNH_2$ (XIV)——This compound was prepared from XIII (525 mg) and Zn dust (165 mg) essentially as described for the preparation of V. Yield 418 mg (96%), mp 148—155 °C, $[\alpha]_D^{21}$ −9.8° (c=1.0, DMF), Rf^1 0.44, Rf^2 0.48, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{43}H_{67}N_7O_{12}$: C, 59.09; H, 7.73; N, 11.22. Found: C, 58.84; H, 7.94; N, 11.46.

Boc–Lys(Z)–Thr(Bzl)–OBzl (XV)——This compound was prepared from H–Thr(Bzl)–OBzl·2 oxalate (2 g), Boc–Lys(Z)–OH·DCHA (3 g), HOBT (743 mg) and WSCI (1.1 g) as described for the preparation of I. The product was reprecipitated from EtOAc with petroleum ether. Yield 2.7 g (79%), mp 84—91 °C, $[\alpha]_D^{21}$ +7.1° (c=1.0, DMF), Rf^1 0.50, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{47}N_3O_8\cdot 2H_2O$: C, 63.69; H, 7.37; N, 6.02. Found: C, 63.73; H, 7.58; N, 5.87.

Boc–Lys(Z)–Lys(Z)–Thr(Bzl)–OBzl (XVI)——This compound was prepared from XV (2.2 g), Boc–Lys(Z)–OH·DCHA (2 g), HOBT (495 mg) and WSCI (703 mg) as described for the preparation of I. The product was precipitated from EtOAc with n-hexane. Yield 2.8 g (90%), mp 72—75 °C, $[\alpha]_D^{21}$ +5.4° (c=1.0, DMF), Rf^1 0.54, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{51}H_{65}N_5O_{11}\cdot 3H_2O$: C, 62.62; H, 7.32; N, 7.16. Found: C, 62.70; H, 7.21; N, 7.04.

Boc–Leu–Lys(Z)–Thr(Bzl)–OBzl (XVII)——This compound was prepared from XVI (1.9 g), Boc–Leu–OH (549 mg), HOBT (297 mg) and WSCI (422 mg) essentially as described for the preparation of I. The product was precipitated from MeOH with ether. Yield 1.5 g (68%), mp 137—143 °C, $[\alpha]_D^{21}$ −6.8° (c=1.0, DMF), Rf^1 0.52, Rf^2 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{76}N_6O_{12}\cdot 2H_2O$: C, 63.79; H, 7.51; N. 7.83. Found: C, 63.81; H, 7.54; N, 7.46.

Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–OBzl (XVIII)——This compound was prepared from XVII (519 mg), Boc–Lys(Z)–OH·DCHA (309 mg), HOBT (74 mg) and WSCI (106 mg) essentially as described for the preparation of XIII. The product was reprecipitated from DMF with H_2O. Yield 497 mg (77%), mp 143—148 °C, $[\alpha]_D^{21}$ −16.1° (c=1.0, DMF), Rf^1 0.59, Rf^2 0.62, single ninhydrin-positive spot. *Anal.* Calcd for $C_{71}H_{94}N_8O_{15}$: C, 65.62; H, 7.29; N, 8.62. Found: C, 65.31; H, 7.45; N, 8.80.

Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–$NHNH_2$ (XIX)——XVIII (433 mg) was dissolved in MeOH–DMF (1 : 1, 8 ml). To this solution, hydrazine hydrate (0.17 ml) was added and the solution was kept standing at 48 °C for 36 h. After evaporation of MeOH, the residue was poured into ice-chilled MeOH with vigorous stirring. The precipitate thereby formed was filtered off and washed with MeOH and H_2O. The dried product was recrystallized from hot MeOH. Yield 364 mg (85%), mp 186—194 °C, $[\alpha]_D^{21}$ −10.4° (c=1.0, DMF), Rf^1 0.42, Rf^2 0.46, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{64}H_{90}N_{10}O_{14}$: C, 62.83; H, 7.42; N, 11.43. Found: C, 62.56; H, 7.69; N, 11.20.

Boc–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–OBzl (XX)——III (1 g) was treated with TFA–anisole (10 ml–2 ml) as described above. The resulting powder was dissolved in DMF (10 ml) containing NMM (0.11 ml). The azide [prepared from 1.8 g of V (1.6 eq)] in DMF (10 ml) and NMM (0.48 ml) were added to the above ice-chilled solution. The mixture, after being stirred at −10 °C for 32 h, was neutralized with a few drops of AcOH and concentrated. The residue was treated with 5% citric acid and the precipitate thereby formed was washed with 5% citric acid and H_2O. The resulting powder was purified by gel-filtration on a Sephadex LH-20 column (2.8 × 100 cm) with DMF containing 3% H_2O. The UV absorption at 280 nm was determined in each fraction (4 ml) and the desired fractions containing a substance of Rf^1 0.61 were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder. Yield 1.2 g (63%), mp 168—177 °C, $[\alpha]_D^{21}$ −14.7° (c=1.0, DMF), Rf^1 0.61, Rf^2 0.68, single chlorine-tolidine-positive spot. *Anal.* Calcd for $C_{105}H_{146}N_{12}O_{25}\cdot 7H_2O$: C, 59.98; H, 7.67; N, 8.00. Found: C, 59.87; H, 7.72; N, 7.91. Amino acid ratios in a 6 N HCl hydrolysate: Ile 1.00, Ser 0.83, Thr 0.86, Glu 4.03, Lys 1.94 (recovery of Ile 82%).

Boc–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–OBzl (XXI)——XX (841 mg) was treated with TFA–anisole (8 ml–1.6 ml) as usual and the TFA salt obtained was dissolved in DMF (8 ml) together with NMM (0.05 ml). To this, X (503 mg, 1.6 eq), HOSu (81 mg) and WSCI (135 mg) were added at 0 °C. After 24 h, the reaction mixture was evaporated and the residue was triturated with 5% citric acid. The powder obtained was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The powder was further purified by reprecipitation from DMF with MeOH. Yield 819 mg (72%), mp 172—179 °C, $[\alpha]_D^{21}$ −18.5° (c=1.0, DMF), Rf^1 0.69, Rf^2 0.73, single chlorine-tolidine-positive spot. *Anal.* Calcd for $C_{139}H_{195}N_{19}O_{33}\cdot 11H_2O$: C, 58.41; H, 7.65; N, 9.39. Found: C, 58.52; H, 7.82; N, 9.11. Amino acid ratios in a 6 N HCl hydrolysate: Leu 1.05, Ile 1.00, Pro 1.87, Ser 0.86, Thr 0.89, Glu 3.94, Asp 0.99, Lys 2.87 (recovery of Ile 84%).

Boc–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr-(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–OBzl (XXII)——The above Boc–(21—38)–OBzl (XXI) (715 mg) was treated with TFA–anisole (7 ml–1.4 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 1, 7 ml) containing NMM (0.028 ml). The azide [prepared from 437 mg of XIV (2 eq)] in DMF–DMSO (1 : 1, 4 ml) and NMM (0.08 ml) were added to the above ice-chilled solution, and the mixture was stirred at −10 °C for 36 h, then neutralized with AcOH and poured into an ice-chilled 5% citric acid with stirring. The precipitate thereby formed was washed with 5% citric acid and H_2O. The dried product was reprecipitated from DMSO with MeOH. Yield 642 mg (71%), mp 181—190 °C, $[\alpha]_D^{21}$ −20.4° (c=1.0, DMSO), Rf^1 0.61, Rf^2 0.64, single chlorine-tolidine-positive spot. *Anal.* Calcd for $C_{177}H_{250}N_{24}O_{43} \cdot 13H_2O$: C, 58.47; H, 7.65; N, 9.25. Found: C, 58.50; H, 7.93; N, 9.18. Amino acid ratios in a 6 N HCl hydrolysate: Leu 1.11, Ile 1.00, Pro 1.82, Ser 0.84, Thr 1.85, Glu 6.84, Asp 0.96, Lys 3.02 (recovery of Ile 85%).

Boc–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OcHex)–Thr(Bzl)–Gln–Glu(OcHex)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OcHex)–Thr(Bzl)–Ile–Glu(OcHex)–Gln–Glu(OcHex)–Lys(Z)–OBzl (XXIII)——The above Boc–(21—38)–OBzl (455 mg) was treated with TFA–anisole (5 ml–1 ml) and the N^{α}-deprotected peptide obtained as described above was dissolved in DMF–DMSO (1 : 1, 5 ml) containing NMM (0.014 ml). The azide [prepared from 306 mg of XIX (2 eq)] in DMF–DMSO (1 : 1, 3 ml) and NMM (0.04 ml) were added to the above ice-chilled solution and the mixture was stirred at −10 °C for 36 h. Additional azide [prepared from 153 mg of hydrazide (1 eq)] in DMF–DMSO (1 : 1, 2 ml) and NMM (0.02 ml) were added and stirring was continued for an additional 24 h. After neutralization with a few drops of AcOH, the solution was concentrated and the residue was treated with 5% citric acid. The resulting powder was washed successively with 5% citric acid, H_2O and MeOH. The dried product was reprecipitated from DMSO with MeOH. Yield 351 mg (59%), mp 192—201 °C, $[\alpha]_D^{21}$ −24.6° (c=1.0, DMSO), Rf^1 0.54, Rf^2 0.50, single chlorine-tolidine-positive spot. *Anal.* Calcd for $C_{236}H_{334}N_{32}O_{55} \cdot 15H_2O$: C, 59.43; H, 7.69; N, 9.40. Found: C, 59.56; H, 7.78; N, 9.25. Amino acid ratios in a 6 N HCl hydrolysate: Leu 2.08, Ile 1.00, Pro 1.87, Ser 0.86, Thr 2.85, Glu 6.92, Asp 0.89, Lys 5.89 (recovery of Ile 81%).

H–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–OH (Corresponding to Positions 16—38 of Thymosin β_4)——The above protected tricosapeptide (159 mg) was treated with 1 M TFMSA–thioanisole in TFA (4 ml) in the presence of $(Me)_2Se$ (100 μl) in an ice-bath for 110 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 30 min and dissolved in 2% AcOH (6 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, 1.5 g) for 30 min, was filtered. The pH of the filtrate was adjusted to 8.5 with 1 N NH_4OH and, after 30 min, to 6.8 with 1 N AcOH and the solution was lyophilized. The powder was dissolved in 2% AcOH (2 ml), applied to a column of Sephadex G-25 (3.6 × 98 cm) and eluted with 2% AcOH. Individual fractions (5 ml each) were collected and the absorbancy at 230 nm was determined. The first peak (tube Nos. 49—56) was collected and the solvent was removed by lyophilization. The residue was dissolved in H_2O (2 ml) and the solution was applied to a column of CM-Sephadex C-25 (2.3 × 65 cm), eluted with a linear gradient formed from 250 ml each of H_2O and 0.07 M NH_4OAc buffer at pH 6.51. Individual fractions (5 ml each) were collected and the absorbancy at 230 nm was determined. A main peak present in the gradient eluates (tube Nos. 36—43) was collected. The solvent was evaporated off and the residue was rechromatographed on a column of CM-Sephadex C-25 as described above. Next, the crude peptide thus obtained was dissolved in a small amount of H_2O and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using the Partridge solvent system as a developing solvent. The zone corresponding to Rf^3 0.11 was separated and extracted with 2% AcOH. The extract was concentrated to a small volume and applied to a column of Sephadex G-25 as described above. Yield 11.4 mg (13%), $[\alpha]_D^{21}$ −64.8° (c=0.4, 3% AcOH), Rf^3 0.11, Rf^4 0.16, single chlorine-tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 3.1. Mobility, 5.3 cm from the origin toward the anode, after running at 2 mA, 650 V for 70 min. The synthetic peptide exhibited a single peak on HPLC using a Cosmosil $5C_{18}$ column (4.6 × 100 mm) at a retention time of 9.89 min, when eluted with a gradient of acetonitrile (32 to 38% in 15 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in 6 N HCl hydrolysate: Leu 2.04, Ile 1.00, Pro 1.81, Ser 0.86, Thr 2.83, Glu 6.85, Asp 0.92, Lys 5.97 (recovery of Ile 84%). Amino acid ratios in AP-M digest: Leu 2.01, Ile 1.00, Pro 1.81, Thr + Gln 4.82 (Calcd as Thr), Asn + Ser 1.81 (Calcd as Ser), Glu 4.87, Lys 6.05 (recovery of Ile 83%).

Fluorometric Blast-Formation Test——A 3 ml aliquot of venous blood was drawn into a syringe containing 25 U/ml of heparin and then mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque–Ficoll gradient.[29] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640 (Gibco) with 10% FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h and then PHA-P was added to each well and incubation was continued under the same conditions for 60 h. T-Lymphocytes in each well were transferred into a test tube and centrifuged for 10 min at 240 g, then the supernatant was removed. A 2 ml aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to Itoh and Kawai.[25]

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References and Notes

1) The amino acid residues mentioned in this paper are of L-configuration except for glycine. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Joint Commission of Biochemical Nomenclature: *Eur. J. Biochem.*, **138**, 9 (1984); *Int. J. Pept. Protein Res.*, **24**, No. 1 (1984). Other abbreviations used: PHA, phytohemagglutinin; DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBT, 1-hydroxybenzotriazole; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; NP, *p*-nitrophenyl; OBzl, benzyl ester; Bzl, benzyl; Troc, β,β,β-trichloroethoxycarbonyl; OcHex, cyclohexyl ester; NMM, *N*-methylmorpholine; OTce, 2,2,2-trichloroethyl ester; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; E-rosette, a rosette with sheep erythrocytes; AcOH, acetic acid; EtOAc, ethyl acetate; TFMSA, trifluoromethanesulfonic acid; TLC, thin-layer chromatography; MeOH, methanol; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; RPMI, Rosewell Park Memorial Institute; FCS, fetal calf serum; ONp, *p*-nitrophenyl ester; CM, carboxylmethyl.
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Reversible Effect of Calcium-Binding Protein on the Ca^{2+}-Induced Activation of Succinate Dehydrogenase in Rat Liver Mitochondoria

MASAYOSHI YAMAGUCHI* and HIROSUKE SHIBANO

Department of Environmental Biochemistry and Toxicology, Shizuoka College of Pharmacy, 2-2-1, Oshika, Shizuoka 422, Japan

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The effect of calcium-binding protein (CaBP) isolated from rat liver cytosol on the Ca^{2+}-induced increase in succinate dehydrogenase activity of rat liver mitochondria was investigated. The liver mitochondrial succinate dehydrogenase activity was significantly increased by addition of Ca^{2+} in the range of 1.0—50 μM. Of various other metals (25 μM) tested, addition of Hg^{2+}, Zn^{2+} and Cd^{2+} caused a significant decrease in succinate dehydrogenase activity of the hepatic mitochondria, while Mg^{2+}, Mn^{2+} and Sr^{2+} had no effect; the increase in the enzyme activity was specific for Ca^{2+}. This Ca^{2+}-induced increase in the mitochondrial succinate dehydrogenase activity was completely blocked by the presence of ruthenium red (10 and 50 μM), an inhibitor of mitochondrial Ca^{2+} uptake, indicating that Ca^{2+} was transported into the mitochondria and activated the enzyme there. In the presence of more than 7.0 μM CaBP, the Ca^{2+} (10 μM)-induced increase in succinate dehydrogenase activity was completely reversed, but 1.4 μM CaBP had no effect on this increase. CaBP itself (15 μM) did not have an inhibitory effect on the basal activity of succinate dehydrogenase. The present results suggest that CaBP regulates the increase in succinate dehydrogenase activity by blocking Ca^{2+} transport into the hepatic mitochondria of rats.

Keywords——calcium-binding protein; calcium ion; zinc ion; cadmium ion; mercuric ion; succinate dehydrogenase; hepatic mitochondria; hepatic cytosol

Introduction

Calcium ion (Ca^{2+}) plays an important role in the regulation of cell function.[1,2] In recent years, it has been demonstrated that liver metabolism is regulated by increase of Ca^{2+} in the cytosol of liver cells due to hormonal stimulation.[3,4] Calmodulin, a Ca^{2+}-binding protein, can amplify the metabolic effect of the cytosolic Ca^{2+} in liver cells; a function of calmodulin is activation of many enzymes.[1,2] Recently, we have reported that a calcium-binding protein (CaBP), which differs from calmodulin, is distributed in the hepatic cytosol of rats.[5-7] This novel protein has a reversible effect on the activation of enzyme by Ca^{2+} in liver cells.[8-10]

In liver cells, the cytosolic Ca^{2+} is transported into the mitochondria and the metal ion stimulates the mitochondrial function.[11,12] Therefore, the present investigation was undertaken to clarify the effect of CaBP on the Ca^{2+}-induced stimulation of the hepatic mitochondrial function. Succinate dehydrogenase is located on the inner membranes of hepatic mitochondria and has an important role in the electron transport system. Here, we report that Ca^{2+} activates succinate dehydrogenase in the hepatic mitochondria, and we show that this Ca^{2+} effect is reversed by CaBP.

Materials and Methods

Animals——Male Wistar rats, weighing 100—120 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.

Reagent——2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, ruthenium red and metallic salts (chloride form) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were of reagent grade. All water used was glass-distilled.

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.[6]

Preparation of Hepatic Mitochondria——The liver was perfused with an ice-cold 0.25 M sucrose solution and immediately cut into small pieces, suspended 1:4 in 0.25 M sucrose solution and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 600 *g* in a refrigerated centrifuge for 10 min and the supernatant was spun at 5500 *g* for 20 min to obtain the mitochondrial fraction.[13] The 5500 *g* pellet was washed twice with ice-cold 0.25 M sucrose solution by centrifugation for 10 min at 5500 *g*. The mitochondrial preparation for enzyme analysis was resuspended in ice-cold distilled water.

Analytical Methods——Enzyme assays were carried out under optimal conditions. Succinate dehydrogenase activity was measured by incubation of the reaction mixture for 15 min at 37 °C in a final volume of 1.0 ml containing 50 mM potassium phosphate (pH 7.4), 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, 50 mM sodium succinate, 25 mM sucrose and the mitochondrial protein (12—18 μg).[14] In separate experiments, the reaction mixture contained 0.1—100 μM Ca^{2+} and/or 10—50 μM ruthenium red or 0.7—15 μM CaBP (final concentrations). The reaction was stopped by addition of trichloroacetic acid (10%, 1.0 ml), then the formazan was extracted with 4.0 ml of ethyl acetate and its absorbance was measured at 490 nm. Enzyme activity was expressed as absorbance at 490 nm per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[15]

Statistical Methods——The significance of differences between values was estimated by using Student's *t*-test. A *p* value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The effect of Ca^{2+} addition on succinate dehydrogenase activity in mitochondria prepared from rat liver is shown in Fig. 1. The mitochondrial succinate dehydrogenase activity was significantly increased by addition of Ca^{2+} in the range of 1.0—50 μM. The maximal increase in the enzyme activity was seen at 10 μM Ca^{2+}. With further increase in the concentration of Ca^{2+}, the increase in succinate dehydrogenase activity declined. At 100 μM Ca^{2+}, the enzume activity showed no increase over the control value.

The effect of various metals on succinate dehydrogenase activity in the hepatic mitochondria is shown in Fig. 2. The enzyme activity was significantly decreased by the presence of Hg^{2+}, Zn^{2+} and Cd^{2+} at a concentration of 25 μM, while Mg^{2+}, Mn^{2+} and Sr^{2+} (25 μM) had no effect on the enzyme activity.

The effect of ruthenium red, a potent inhibitor of Ca^{2+} transport into the mitochondria,[16] on the Ca^{2+}-induced increase in the mitochondrial succinate dehydrogenase activity is shown in Fig. 3. The presence of 10 and 50 μM ruthenium red had no inhibitory effect on the basal activity of succinate dehydrogenase. The Ca^{2+}-induced increase in the enzyme activity,

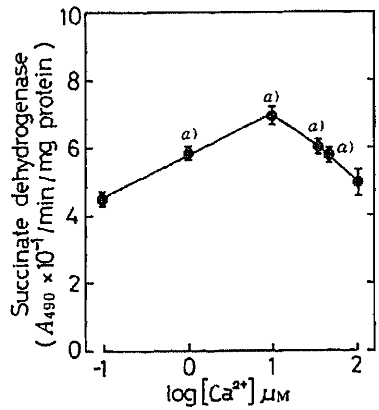

Fig. 1. Effect of Increasing Concentrations of Ca^{2+} on the Activity of Succinate Dehydrogenase in the Hepatic Mitochondria of Rats

Addition of 10^{-1} μM Ca^{2+} did not affect 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+} addition.

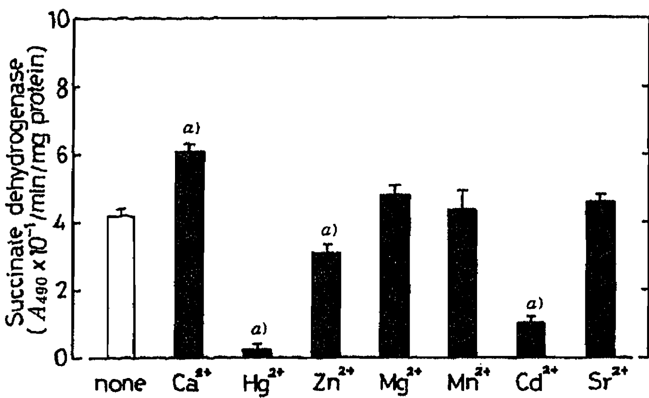

Fig. 2. Effect of Various Metals on Succinate Dehydrogenase Activity in the Hepatic Mitochondria of Rats

The enzyme activity was measured in the reaction mixture containing 25 μM metal as a final concentration. Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the control (none) value.

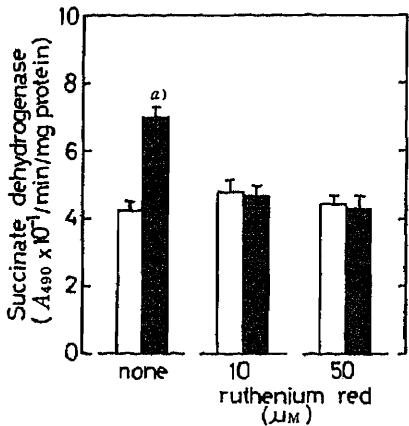

Fig. 3. Effect of Ruthenium Red on the Increase of Succinate Dehydrogenase Activity Caused by Ca^{2+} Addition in the Hepatic Mitochondria of Rats

The enzyme activity was measured in the reaction mixture containing 10 and 50 μM ruthenium red as a final concentration in the presence or absence of 10 μM Ca^{2+}. Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the control value. □, control; ■, 10 μM Ca^{2+} addition.

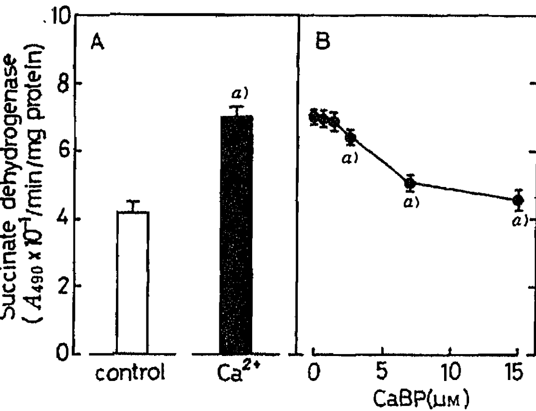

Fig. 4. Effect of Calcium-Binding Protein (CaBP) on the Ca^{2+}-Induced Increase of Succinate Dehydrogenase Activity in the Hepatic Mitochondria of Rats

Figure A shows the effect of 10 μM Ca^{2+} addition on succinate dehydrogenase activity. Figure B shows the effect of increasing concentrations of CaBP (0.7, 1.4, 2.6, 7.0 and 15 μM). Each value represents the mean $\pm$ S.E.M. of 5 experiments. *a*) $p<0.01$, as compared with the value obtained from 10 μM Ca^{2+} addition.

however, was completely blocked by the presence of ruthenium red (10 and 50 μM).

The effect of CaBP isolated from rat liver cytosol on the Ca^{2+}-induced increase in the mitochondrial succinate dehydrogenase activity is shown in Fig. 4. Addition of Ca^{2+} (10 μM) caused a significant increase of succinate dehydrogenase in the hepatic mitochondria (Fig. 4A). This increase was completely reversed by the presence of CaBP at the concentration of 7.0 μM (Fig. 4B). This effect of CaBP was saturated at 15.0 μM. In the presence of less than 1.4 μM CaBP, the reversible effect was not seen. The effect of CaBP (7.0 μM) was also observed at 25 μM Ca^{2+} (data not shown). CaBP itself (15 μM) did not have an inhibitory effect on the basal activity of succinate dehydrogenase; the basal activity was 4.21 ± 0.23 ($A_{490} \times 10^{-1}$/min/mg protein) and in the case of CaBP addition the enzyme activity was 4.32 ± 0.34 ($A_{490} \times 10^{-1}$/min/mg protein) in five experiments.

Discussion

Succinate dehydrogenase located on the inner membranes of hepatic mitochondria is physiologically important in electron transport and oxidative phosphorylation. This enzyme activity was significantly increased by addition of Ca^{2+} in the range of 1.0–50 μM; the maximal increase was seen at 10 μM Ca^{2+}. Of the various divalent ions used, Ca^{2+} uniquely increased succinate dehydrogenase activity in the hepatic mitochondria; the enzyme activity was markedly decreased by addition of Hg^{2+}, Zn^{2+} and Cd^{2+} (25 μM). These results indicate that the mitochondrial succinate dehydrogenase is regulated by Ca^{2+} in the physiological concentration range, although the mechanism by which Ca^{2+} activates the enzyme is unknown. The present finding, that Ca^{2+} can activate the mitochondrial succinate dehydrogenase, suggests a role of Ca^{2+} in the regulation of the mitochondrial function.

Ruthenium red is a potent inhibitor of the transport of Ca^{2+} into the hepatic mitochondria.[16] The presence of ruthenium red (10 and 50 μM) completely prevented the increase in the mitochondrial succinate dehydrogenase activity by addition of Ca^{2+} (10 μM); ruthenium red had no effect on the basal activity of the enzyme. This phenomenon may be a result of inhibition of Ca^{2+} transport into the hepatic mitochondria by ruthenium red, since Ca^{2+} added to the enzyme reaction mixture is transported into the hepatic mitochondria in the presence of succinate as the substrate. In fact, the presence of 1 and 3 mM adenosine triphosphate (ATP) did not enhance the increase in the mitochondrial succinate dehydrogenase activity caused by Ca^{2+} addition (data not shown), although 3 mM ATP stimulates the transport of Ca^{2+} into the hepatic mitochondria of rats.[17] Presumably, Ca^{2+} is transported into the hepatic mitochondria and activates succinate dehydrogenase in the inner membranes of mitochondria.

CaBP isolated from hepatic cytosol of rats reverses the activation of liver cytosolic enzymes by Ca^{2+} at a cell physiological level.[8–10] If CaBP disturbs the transport of Ca^{2+} into the hepatic mitochondria, the increase in the mitochondrial succinate dehydrogenase activity caused by Ca^{2+} addition may be weakened by the presence of CaBP in the enzyme reaction mixture. In fact, the increase in succinate dehydrogenase activity caused by Ca^{2+} (10 μM) was completely blocked by the presence of more than 7.0 μM CaBP. This effect of CaBP was not seen at a concentration of less than 1.4 μM. In the previous report,[17] it was shown that the mitochondrial transport of Ca^{2+} in the presence of 1.4 μM CaBP was appreciably increased by addition of 3 mM ATP. It is possible that CaBP at higher concentrations can prevent the transport of Ca^{2+} into the mitochondria, because of binding of Ca^{2+} by the protein. The molecular weight of CaBP was estimated to be 28800, and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[7] This protein has 6—7 high affinity binding sites per molecule.[7] CaBP may bind Ca^{2+} in the extramitochondrial region and weaken the effect of the metal on succinate dehydrogenase by blocking its transport into the hepatic mitochondria.

Thus far, it has been demonstrated that CaBP has a reversible effect on the activation of various enzyme by Ca^{2+} in the liver cells of rats.[8–10] CaBP differs from calmodulin, which modulates many biochemical effects of Ca^{2+} in cells.[1,2] CaBP may play a cell physiological role by controlling the stimulation of liver cell functions by Ca^{2+}, especially in the cytosol. It is proposed that CaBP is an important regulatory protein for liver cytosolic Ca^{2+}. Further investigations are needed to clarify the role of CaBP in liver cells.

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Properties of Adenosine Triphosphate-Hydrolyzing Enzymes in Membrane Vesicles of *Vibrio parahaemolyticus*

YUKI SAKAI, YUKIKO SUMIDA, MASAAKI TSUDA, SUMIO SHINODA, and TOMOFUSA TSUCHIYA*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka, Okayama 700, Japan

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The properties and roles of adenosine triphosphate (ATP)-hydrolyzing enzymes in the membrane of *Vibrio parahaemolyticus* were investigated. At least two (perhaps three) types of ATP-hydrolyzing enzyme was present in everted membrane vesicles. The adenosine triphosphatase (ATPase) activity of one of the enzymes was sensitive to dicyclohexylcarbodiimide and the activity was lost when the membranes were washed with buffer containing ethylenediaminetetraacetic acid. This enzyme seems to be an H^+-translocating ATPase. The other ATP-hydrolyzing activity was due to a tightly bound enzyme that could not be removed from the membranes by the washing. This enzyme required higher concentrations of Mg^{2+} (10 to 20 mM) and Cl^- (100 mM) for maximal activity. Zn^{2+} strongly inhibited the activity. The substrate specificity of this enzyme activity showed that it was a 5′-nucleotidase.

Keywords——membrane-bound ATPase, *V. parahaemolyticus*; H^+-ATPase; 5′-nucleotidase; Na^+-ATPase

Membrane-bound adenosine triphosphatases (ATPases) play important roles in energy transduction and cation transport. The H^+-translocating ATPase is involved in adenosine triphosphate (ATP) synthesis and hydrolysis, both of which are coupled to H^+ translocation across the membrane.[1] The H^+-translocating ATPase is believed to be the most fundamental type of ion-translocating ATPase, and to be ubiquitous in nature.[2] A K^+-translocating ATPase has been found in *Escherichia coli* membranes,[3] and an Na^+-translocating (perhaps Na^+, K^+-translocating)-ATPase in *Streptococcus faecalis*.[4] Recently, a K^+-ATPase, which has been called ktrI, has also been found in *S. faecalis*.[5] Thus there may be other as yet unidentified cation(or anion)-translocating ATPase in microbial cell membranes.

We are interested in ion transport in *Vibrio parahaemolyticus*, which is a major cause of food poisoning in Japan. Although the pathogenicity of this organism is well understood,[6] the physiological and biochemical properties have not been analyzed well. This organism requires about 0.2 M NaCl for maximal growth. We have reported that the respiratory chain of this organism extrudes Na^+,[7] like that of *V. alginolyticus*.[8] Influx of Na^+ down the electrochemical potential is utilized to drive transport of nutrients in several species of *Vibrio*.[7,9,10] Furthermore, influx of Na^+ is also utilized for ATP synthesis[11] and flagella rotation in *V. alginolyticus*.[12] Thus, the significance of Na^+ circulation in membrane energy transduction in *Vibrio* has been partly clarified. However, the significance of Cl^- in membrane processes or energy transduction in *V. parahaemolyticus* is not well understood. There are many ion-translocating ATPase in biological membranes, and in this work we examined the characteristics of the ATP-hydrolyzing activities in the membrane fraction of *V. parahaemolyticus* in order to obtain information on the roles of the enzymes.

Experimental

Bacterium and Growth——*V. parahaemolyticus* AQ3334 cells were grown aerobically at 37 °C in medium S consisting of 50 mM Tris–HCl (pH 7.8), 25 mM $MgSO_4$, 10 mM KCl, 1 mM $CaCl_2$, 0.33 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM $(NH_4)_2SO_4$ and 0.2 M NaCl, supplemented with 0.5% polypeptone.

Preparation of Membrane Vesicles——Cells were harvested in the late-exponential phase of growth, washed twice with 0.3 M 4-morpholinepropanesulfonic acid (Mops)–KOH (pH 7.5) containing 5 mM $MgSO_4$ and suspended in the same buffer. Vesicles were prepared in a French press,[7] washed twice with the same buffer and stored at −80 °C. Ethylenediaminetetraacetic acid (EDTA)-treated membrane vesicles were prepared by washing vesicles from the French press with 3 mM *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)–Tris (pH 8.0) containing 0.5 mM EDTA and 1 mM 2-mercaptoethanol.

Assay of ATP-Hydrolyzing Activities——The standard reaction mixture (0.6 ml) contained 20 mM Tricine–Tris (pH 8.0), 2 mM (or 20 mM) $MgSO_4$, 0.2 M (or 0.1 M) NaCl and membrane vesicles (about 5 μg protein). Additions were made where indicated. After the addition of 4 mM ATP or other nucleotides, the reaction mixture was incubated at 37 °C for 5 min. The inorganic phosphate released was determined colorimetrically.[13] One unit of activity is defined as the amount releasing 1 μmol of inorganic phosphate per min at 37 °C.

Protein Determination——Protein concentration was determined by a published procedure.[14]

Results

Membrane-Bound ATP-Hydrolyzing Activities

The ATP-hydrolyzing activity (ATPase activity) in membrane vesicles was greatly enhanced by Mg^{2+}, which was maximally effective at 10 to 20 mM (Fig. 1). Some of this activity was probably due to the H^+-translocating ATPase. Since the optimum ratio of ATP to Mg^{2+} is 2 for the H^+-translocating ATPase of *E. coli*,[15] ATP hydrolysis by the H^+-translocating ATPase is probably maximum at about 2 mM Mg^{2+}, since the concentration of ATP in the assay mixture was 4 mM (ATP : Mg^{2+} = 2 : 1). If this is the case, then the ATPase activity observed at higher concentrations of Mg^{2+} should be due to some other enzyme(s) than the H^+-translocating ATPase. This notion was confirmed by the following experiments. When membrane vesicles (everted) were washed with buffer containing EDTA, which is an easy way to remove the F_1 portion (catalytic portion) of the H^+-translocating ATPase of *E. coli*,[16] the ATPase activity observed at 2 mM Mg^{2+} was greatly reduced, but most of the ATPase activity observed at 10 to 20 mM Mg^{2+} was retained. This implies that the ATPase activity observed at higher Mg^{2+} concentrations was due to an enzyme(s) that was tightly bound to the membranes. We were able to solubilize the ATP-hydrolyzing enzyme(s) with a

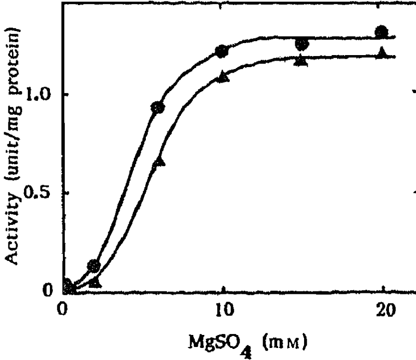

Fig. 1. Effect of Mg^{2+} on ATP-Hydrolyzing Activity in Membrane Vesicles

Assays were performed as described in the text. Various concentrations of Mg^{2+} were added to assay mixtures containing 0.2 M NaCl and either untreated membrane vesicles (●) or EDTA-treated membrane vesicles (▲).

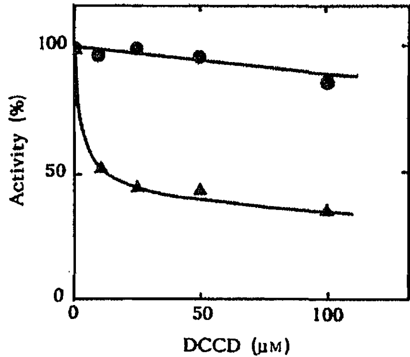

Fig. 2. Effect of DCCD on ATP-Hydrolyzing Activity

Various concentrations of DCCD were added to assay mixtures containing membrane vesicles (untreated) and either 2 mM (▲) or 20 mM Mg^{2+} (●). The ATP-hydrolyzing activities of uninhibited vesicles (100%) were 0.084 and 0.86 unit/mg of protein at 2 mM and 20 mM Mg^{2+}, respectively.

non-ionic detergent, heptylthioglucoside (unpublished observation). Thus, the enzyme(s) seems to be an integral membrane protein.

We found previously that H^+-translocation in membrane vesicles of *V. parahaemolyticus* is elicited by ATP hydrolysis.[7] Such H^+-translocation was maximum at 2 mM Mg^{2+}, and was not observed in EDTA-washed membranes (data not shown). Thus, the F_1 portion of the H^+-translocating ATPase seems to be removed from the membranes by washing them with buffer containing EDTA. In fact, two protein bands that seemed to correspond to the α and β subunits of F_1 were observed when the supernatant fraction of the EDTA-wash was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

To distinguish the two ATPase activities, we tested the effect of dicyclohexylcarbodiimide (DCCD), a commonly used inhibitor of the H^+-translocating ATPase, on the two activities. As expected, DCCD inhibited the activity measured at 2 mM Mg^{2+}, but had little effect on that measured at 20 mM Mg^{2+} (Fig. 2). Azide, which is a potent inhibitor of F_1 of *E. coli*, did not inhibit the ATPase activity at either 2 mM Mg^{2+} or 20 mM Mg^{2+} (data not shown).

The pH optimum for the ATP-hydrolyzing activity was about 8.0 with either 2 mM Mg^{2+} or 20 mM Mg^{2+} (data not shown).

Effects of Monovalent Cations and Anions and Divalent Cations

We tested the effects of monovalent cations and anions of the ATP-hydrolyzing activity at both 2 mM and 20 mM Mg^{2+} (Table I). At 2 mM Mg^{2+}, Na^+, NH_4^+, Li^+ and K^+ increased the ATP-hydrolyzing activity about 10 times, the strengths of their effects being in that order. We also tested the effects of anions; Cl^-, SO_4^{2-} and CH_3COO^- enhanced the activity, while NO_3^- was less effective. Thus a certain level of ionic strength, but not of particular ions, seems to be necessary for the activity of the H^+-translocating ATPase. It should be pointed out, however, that we observed significantly higher activity of ATP hydrolysis with Na^+ than with other monovalent cations.

At 20 mM Mg^{2+}, Cl^- and NO_3^- ($Cl^- > NO_3^-$) greatly stimulated the ATP-hydrolyzing activity, but SO_4^{2-} and CH_3COO^- had little effect. Since *V. parahaemolyticus* lives under Cl^--rich conditions, Cl^- would be the natural activator of the ATP-hydrolyzing enzyme.

We then tested the effect of Cl^- concentration on the ATP-hydrolyzing activity at 20 mM Mg^{2+} (Fig. 3). With increasing concentrations of Cl^- up to 100 mM, we observed higher activity. No further stimulation of ATP hydrolysis by Cl^- was observed at higher con-

Table I. Effects of Monovalent Cations and Anions on ATP Hydrolysis by Membrane Vesicles

Salt	ATP-hydrolyzing activity (units/mg protein)	
	At 2 mM $MgSO_4$	At 20 mM $MgSO_4$
—	0.04	0.32
NaCl	0.43	2.58
KCl	0.22	2.15
LiCl	0.33	2.49
NH_4Cl	0.40	2.26
$NaNO_3$	0.13	1.71
$NaOCOCH_3$	0.43	0.55
Na_2SO_4	0.46	0.54

Hydrolysis of ATP was measured with membrane vesicles (untreated). Salts were added to assay mixtures at 0.2 M with 2 mM $MgSO_4$ or at 0.1 M with 20 mM $MgSO_4$ to obtain maximum activities.

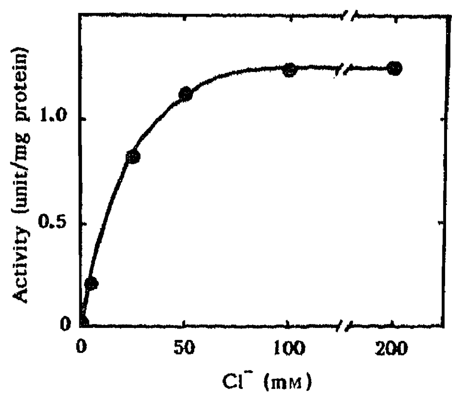

Fig. 3. Effect of Cl^- Concentration on ATP-Hydrolyzing Activity

Hydrolysis of ATP was measured in the presence of membrane vesicles (untreated), 20 mM $MgSO_4$ and various concentrations of Cl^- Total concentration of NaCl plus Na_2SO_4 in assay mixtures was 0.2 M.

TABLE II. Effects of Divalent Cations on ATP Hydrolysis

Salt	ATP-hydrolyzing activity (units/mg protein)	
	At 2 mM salt	At 20 mM salt
—	0.02	0.05
$MgCl_2$	0.11	1.58
$CaCl_2$	0.12	0.63
$MnCl_2$	0.06	0.52
$CoCl_2$	0.13	0.28
$FeSO_4$	0.02	0.12
$ZnSO_4$	0.04	0.14
$NiCl_2$	0.02	0.06

Hydrolysis of ATP was measured with membrane vesicles (untreated). Assay mixtures contained either 2 mM or 20 mM salts as indicated, and 0.2 M NaCl.

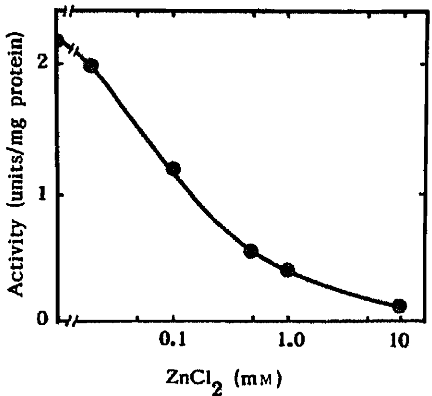

Fig. 4. Inhibition of the 5′-Nucleotidase by Zn^{2+}

The ATP-hydrolyzing activity in membrane vesicles (untreated) was measured in the presence of 20 mM $MgSO_4$, 0.1 M NaCl and various concentrations of $ZnCl_2$.

TABLE III. Substrate Specificity of Membrane-Bound 5′-Nucleotidase

Substrate	Hydrolyzing activity (units/mg protein)
ATP	1.93
GTP	1.43
CTP	0.80
UTP	1.27
ADP	1.74
AMP	1.71
3′-CMP	<0.01

Hydrolysis of nucleotides was measured with membrane vesicles (untreated) in the presence of 20 mM $MgSO_4$ and 0.1 M NaCl. All nucleotides except cytidine-3′ monophosphate (3′-CMP) were 5′-nucleotides.

centrations than 100 mM.

Among the divalent cations tested, Mg^{2+}, Ca^{2+} and Co^{2+} stimulated the ATP-hydrolyzing activity to almost the same extent at 2 mM (Table II). On the other hand, Mg^{2+} caused great stimulation, Ca^{2+} and Mn^{2+} considerable stimulation, and Co^{2+} slight stimulation at concentrations of 20 mM.

The ATP-hydrolyzing activity observed at 20 mM Mg^{2+} was strongly inhibited by Zn^{2+} (Fig. 4). We also observed inhibition by Ni^{2+} (data not shown). The inhibition by Zn^{2+} was greater than that by Ni^{2+}, 50% inhibition being observed at about 0.1 mM Zn^{2+} and 4 mM Ni^{2+}. The ATPase activity observed at 2 mM Mg^{2+} was insensitive to Zn^{2+} and Ni^{2+} (data not shown).

Substrate Specificity

Since the ATP-hydrolyzing activity observed at 10 to 20 mM Mg^{2+} was stimulated by Cl^-, this activity seemed to be that of anion-stimulated 5′-nucleotidase which has been reported in *V. alginolyticus*.[17] Very similar membrane-bound 5′-nucleotidase has been reported in *V. costicola*.[18] Thus, we tested the substrate specificity of the enzyme. With 20 mM Mg^{2+}, ATP, guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytidine triphosphate (CTP) (ATP > GTP > UTP > CTP) were all hydrolyzed, and adenosine di-

phosphate (ADP) and adenosine monophosphate (AMP) were also hydrolyzed (Table III). Although all the 5′-nucleotides tested were hydrolyzed, 3′-nucleotide was not. Therefore, we concluded that the ATP-hydrolyzing activity observed at 10 to 20 mM Mg^{2+} was mostly due to anion (Cl^-)-stimulated 5′-nucleotidase.

Discussion

The respiratory chain of *Vibrio* extrudes both H^+ and Na^+.[8] We reported previously that H^+-translocating ATPase is present in cell membranes of *V. parahaemolyticus*.[7] Thus, it seemed that respiration-linked ATP synthesis catalyzed by the H^+-translocating ATPase occurs in this organism too. As described in this paper, the properties of this ATPase were similar to those of the H^+-translocating ATPase of *E. coli*; namely, the enzyme required Mg^{2+}, and was inhibited by DCCD, and the activity was lost from the membranes when the membranes were washed with buffer containing EDTA.

We thought that there might be an Na^+-translocating ATPase in addition to the H^+-translocating ATPase in the membranes of *V. parahaemolyticus*, and that it might synthesize ATP by using the electrochemical potential of Na^+ established by the respiratory chain or the Na^+/H^+ antiporter, which is able to convert an electrochemical potential of H^+ to that of Na^+. If such an Na^+-translocating ATPase is present in membranes of this organism, the membrane-bound ATPase activity should be stimulated by Na^+. In fact, we detected some membrane-bound ATPase activity at 2 mM Mg^{2+}, that was stimulated by Na^+. Although it showed rather broad ion specificity, the ATP-hydrolyzing activity was obviously higher in the presence of Na^+ than of other monovalent cations (Table I, at 2 mM Mg^{2+}). Its activity was lower in the presence of $NaNO_3$, suggesting that NO_3^- was inhibitory. Since the H^+-translocating ATPase functions well at 2 mM Mg^{2+}, most of the ATP-hydrolyzing activity observed at this Mg^{2+} concentration seems to be due to this enzyme. However, the fact that DCCD inhibited only 50—60 % of the ATPase activity at 2 mM Mg^{2+} suggests the presence of some other ATPase(s). Very recently, Dibrov *et al.* reported the presence of "Na^+-ATP-synthase" in *V. alginolyticus*.[11] They observed Na^+-driven ATP synthesis. However, the biochemical properties of the "Na^+-ATP-synthase" are not known. Thus, the presence of Na^+-ATPase in *V. parahaemolyticus* seems to be likely. However, it is difficult to characterize such an ATPase even if it is present, because *V. parahaemolyticus* membranes have very strong ATP-hydrolyzing activity due to Cl^--stimulated 5′-nucleotidase. Therefore, we are now trying to isolate mutants defective in Cl^--stimulated 5′-nucleotidase to characterize the Na^+-stimulated ATPase. We are also testing whether or not ATP is synthesized when an artificial electrochemical potential due to Na^+ is imposed across the cell membrane of *V. parahaemolyticus*.

We observed the 5′-nucleotidase activity in both everted membrane vesicles and whole cells of *V. parahaemolyticus* (manuscript in preparation). It has been reported in *V. costicola* that 5′-nucleotidase is measurable with whole cells.[19] The outwardly-oriented 5′-nucleotidase and the inwardly-oriented 5′-nucleotidase seem to be the same enzyme, because they showed very similar properties, Cl^--dependency, Mg^{2+}-dependency, substrate specificity and effect of inhibitors. This suggests that the substrate could reach the catalytic site of the enzyme from either side of the membrane, because it is unreasonable to suppose that ATP, a substrate of the enzyme, can penetrate through the cell membrane of this organism. We found previously that membranes prepared by the French press method are everted vesicles.[7] It is unlikely that they are a mixture of everted and right-side-out membrane vesicles, because their ATP-hydrolyzing activity was not increased when they were made permeable by toluene treatment (data not shown). Thus, ATP and other 5′-nucleotides may be hydrolyzed by the 5′-nucleotidase even at the inner surface of the cytoplasmic membrane, although if so, there must

be a proper control mechanism to prevent waste of 5′-nucleotides. The outwardly-oriented 5′-nucleotidase activity seems to be involved in utilization of extracellular ATP and other nucleotides. This enzyme cleaves 5′-nucleotides to nucleosides, and then nucleosides would be taken up by the cells. It should be pointed out that 5′-nucleotides have been reported to be present in sea water, although at low level.[20]

Why is the Cl^--stimulated 5′-nucleotidase an integral membrane protein? What is the role of inwardly-oriented 5′-nucleotidase activity? One possibility is that this 5′-nucleotidase functions as a Cl^- pump which utilizes intracellular 5′-nucleotides, perhaps ATP. Another possibility is an involvement of this enzyme in metabolism of 5′-nucleotides on the cytoplasmic side. We characterized the 5′-nucleotidase in *V. parahaemolyticus* to obtain insight into the role of Cl^- in membrane processes in this microorganism. Transport of Cl^- may take place as a result of hydrolysis of 5′-nucleotides, especially ATP, because many ion-translocating ATPases are known in biological membranes, and these ATPases are stimulated by the ions transported. Studies are required on Cl^- transport as well as Na^+ transport in *Vibrio*, because this organism requires NaCl for growth. Our preliminary results indicated that *V. parahaemolyticus* has a Cl^- transport system(s) (unpublished observation). We are now investigating the relationship between Cl^- transport and the Cl^--stimulated 5′-nucleotidase.

The ATP-hydrolyzing activity of membranes (specific activity expressed as units/mg protein) of *V. parahaemolyticus* varied from membrane preparation to preparation. This suggests the presence of several types of ATPase with different properties (stability, activation or inhibition by various factors, inducibility and so on). It is important to characterize these membrane ATPases of *V. parahaemolyticus* to get insight into their roles and into the membrane energetics of this organism.

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High-Performance Affinity Adsorbents for Trypsin-Family Enzymes Prepared with TSKgel G3000PW

AKIKO KANAMORI, NOBUKO SENO and ISAMU MATSUMOTO*

Department of Chemistry, Faculty of Science, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112, Japan

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For high-performance liquid affinity chromatography (HPLAC) of trypsin-family enzymes, two kinds of adsorbents (I and II) were prepared by convenient procedures using *m*-aminobenzamidine (ABA) and TSKgel G3000PW, a carrier for high-performance gel chromatography. For adsorbent I, ABA was immobilized on succinyl-TSKgel G3000PW, which was prepared by successive epoxy-activation, amination and succinylation of TSKgel G3000PW with the aid of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (about 16 μmol ABA/g wet gel, about 80% yield). For adsorbent II, ABA was immobilized on formyl-carrier, which was prepared by successive epoxy-activation, hydrolysis and periodate-oxidation of TSKgel G3000PW through reductive amination with sodium cyanoborohydride (19 μmol ABA/g wet gel, 98% yield). In the case of HPLAC on adsorbent I, bovine and *Streptomyces griseus* trypsins were eluted at 12 and 19 min, respectively, whereas the enzymes were retained on adsorbent II and were recovered efficiently with eluting buffer containing 6-aminohexanoic acid. The results indicate that adsorbent I is useful for rapid analysis of the enzymes and adsorbent II for the preparation of the enzymes.

Keywords——affinity adsorbent; high-performance liquid affinity chromatography; trypsin; trypsin-family enzyme; *m*-aminobenzamidine; 6-aminohexanoic acid

Introduction

We have reported convenient procedures to prepare high-capacity affinity adsorbents for trypsin.[1] *m*-Aminobenzamidine (ABA), an affinity ligand for trypsin-family proteases,[2] was immobilized on two types of formyl carriers by reductive amination. Formyl carriers having long spacers were prepared by periodate oxidation of glucose carriers obtained by coupling glucose to epoxy-activated carriers. Formyl carriers having short spacers were prepared by periodate oxidation of glyceryl carriers obtained by hydrolysis of epoxy-activated carriers. High concentrations of ABA (15—21 μmol per g wet gel) were efficiently immobilized on both types of carriers. Regardless of the length of the spacers, adsorbents prepared with Sepharose gel exhibited extremely high adsorption capacities, *e.g.*, about 30 mg of bovine trypsin per ml of gel. Though the adsorbents prepared with Fractogel TSK, a hydrophilic vinyl polymer matrix, could be used for high-performance liquid affinity chromatography (HPLAC) at a flow-rate of 0.5 ml/min, the results were not completely satisfactory because some tailing occurred. Ito *et al.* have previously prepared an adsorbent for HPLAC of trypsins, using Asahipak GS-gel, a micro-particulate polyvinyl alcohol gel.[3] After the hydroxyl groups had been activated with 1,1′-carbonyldiimidazole, 6-aminohexanoic acid was coupled as a spacer, then ABA was immobilized on the spacer. The adsorbents thus prepared could be used to analyze microgram amounts of bovine trypsin in less than 1 h.

In this study, we prepared two types of adsorbents for HPLAC of trypsins using formyl and carboxyl derivatives of TSKgel G3000PW, which is a hydrophilic polyacrylate matrix suitable for high-performance gel permeation liquid chromatography. ABA was immobilized

on the formyl carrier *via* an alkylamine bond and on the carboxyl carrier *via* an amide bond. The adsorbents thus obtained were successfully used for rapid analysis or preparation of trypsins: the retention time of bovine trypsin was 12—16 min.

Experimental

Materials——TSKgel G3000PW (molecular weight exclusion limit 5×10^4 for protein, particle size 8—15 μm) was obtained from Toyo Soda Manufacturing Co. (Tokyo, Japan), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), 6-aminohexanoic acid (AHA), sodium borohydride ($NaBH_4$), sodium metaperiodate ($NaIO_4$), casein and trypsin (2000 units/g) from Wako (Osaka, Japan), bovine serum albumin from Sigma, sodium cyanoborohydride ($NaCNBH_3$) from Nakarai (Kyoto, Japan), *m*-aminobenzamidine (ABA) dihydrochloride monohydrate from Aldrich, benzoyl-L-arginine ethyl ester (BAEE) from Fluka, pronase E from Kaken (Tokyo, Japan) and Toyo membrane filters from Toyo Roshi (Tokyo, Japan). Lysozyme was isolated from egg white by the direct crystallization procedure.[4]

Preparation of Affinity Adsorbents——Two kinds of adsorbents I and II were prepared by the routes shown in Chart 1. TSKgel G3000PW was activated with epichlorohydrin as described previously for Toyopearl HW-65.[5]

Route 1: Succinyl-TSKgel G3000PW was obtained by amination of epoxy-activated gel with concentrated ammonia solution and subsequent succinylation of the amino derivative with succinic anhydride as described

epichlorohydrin

route 1 route 2

NH_4OH NaOH, $NaBH_4$

$NaIO_4$

EEDQ
ABA

ABA
$NaCNBH_3$
$NaBH_4$

ABA-TSKgel G3000PW I ABA-TSKgel G3000PW II

Chart 1. Preparation of Trypsin Adsorbents

previously.[5] A solution of EEDQ (120 mg) in ethanol (4 ml) was added to a suspension of succinyl-TSKgel G3000PW (8 g) in water (4 ml). Then ABA (36 mg) was added and the whole was shaken for 24 h at 25 °C. The gel was washed sequentially with 40 ml of 50% ethanol, 30 ml of 1 M NaCl and 80 ml of water.

Route 2: The epoxy-activated TSKgel G3000PW (7 g) was suspended in 140 ml of 0.1 M NaOH containing 14 mg of $NaBH_4$ and incubated for 24 h at 40 °C with shaking. After being washed with water until epoxy groups were not detectable in the washings according to the method of Sundberg and Porath,[6] the gel (6 g) was suspended in 9 ml of 0.1 M $NaIO_4$ and shaken for 1 h at 4 °C. After being washed with water and 15 mM phosphate-buffered saline (PBS), pH 7.0, formyl carrier thus obtained was suspended in 12 ml of PBS containing 27 mg of ABA, and incubated with shaking at 4 °C for 24 h. After addition of 30 mg of $NaCNBH_3$, the suspension was shaken at 4 °C for 6 h. The gel was washed successively with PBS and water, and then treated with 30 mg of $NaBH_4$ at 4 °C for 15 h to convert the remaining formyl groups into hydroxymethyl groups. The amount of immobilized ABA was determined by difference analysis of the absorbance at 320 nm ($\varepsilon_{320\,nm} = 1.80 \times 10^3$ l mol^{-1} cm^{-1} in 15 mM PBS, pH 7.0) of the combined filtrate and washings.

Chromatographic System——ABA-TSKgel G3000PW I and II were packed into 7.5 mm i.d. × 7.5 cm stainless columns by the slurry packing method at a flow rate of 2 ml/min. An HPLC system with a high-pressure pump (model HLC-803D, Toyo Soda) and a spectromonitor (UV-8000, Toyo Soda) was used.

Affinity Chromatography of Trypsins——Crude bovine trypsin (630 mg) was dissolved in 10 ml of 0.05 M Tris–HCl containing 0.2 M NaCl and 0.02 M $CaCl_2$, pH 7.8 (eluent 1), and the solution was centrifuged at 20000 *g* for 30 min. The supernatant was filtered through a Toyo membrane filter with a pore size of 0.45 μm. A solution of pronase E (100 mg) in eluent 1 (4 ml) was also filtered through the membrane. A 100-μl aliquot of bovine trypsin or pronase E solution was applied to the columns at a flow rate of 1 ml/min at room temperature. The mobile phase was eluent 1. Conditions of the elution of the trypsins are given in the results section. Trypsin and pronase activities were determined with BAEE and casein as substrates, respectively.[1]

Results

Preparation of Affinity Adsorbents

In the case of adsorbent prepared with the carboxyl carrier (adsorbent I), the concentration of immobilized ABA was roughly estimated to be 16 μmol/g wet gel (about 80% yield). In the case of adsorbent prepared with formyl carrier (adsorbent II), the ligand

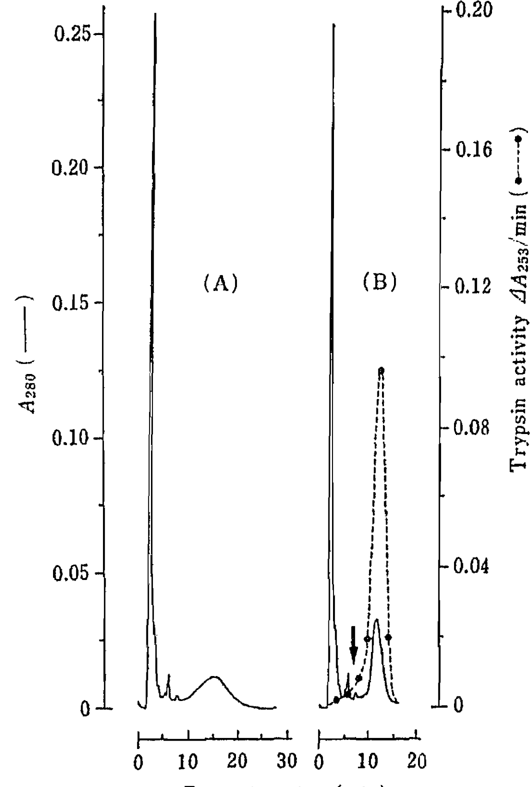

Fig. 1. HPLAC of Bovine Trypsin on ABA-TSKgel G3000PW I

Crude trypsin solution (100 μl) in 0.05 M Tris–HCl–0.2 M NaCl–20 mM $CaCl_2$, pH 7.8 (eluent 1), (A_{280} = 2.6) was loaded on the column (7.5 mm i.d. × 7.5 cm) at a flow rate of 1 ml/min at room temperature. The elution was performed isocratically in A. The eluent was changed at the point indicated by the arrow to eluent 1 + 20 mM AHA (pH 7.8) in B.

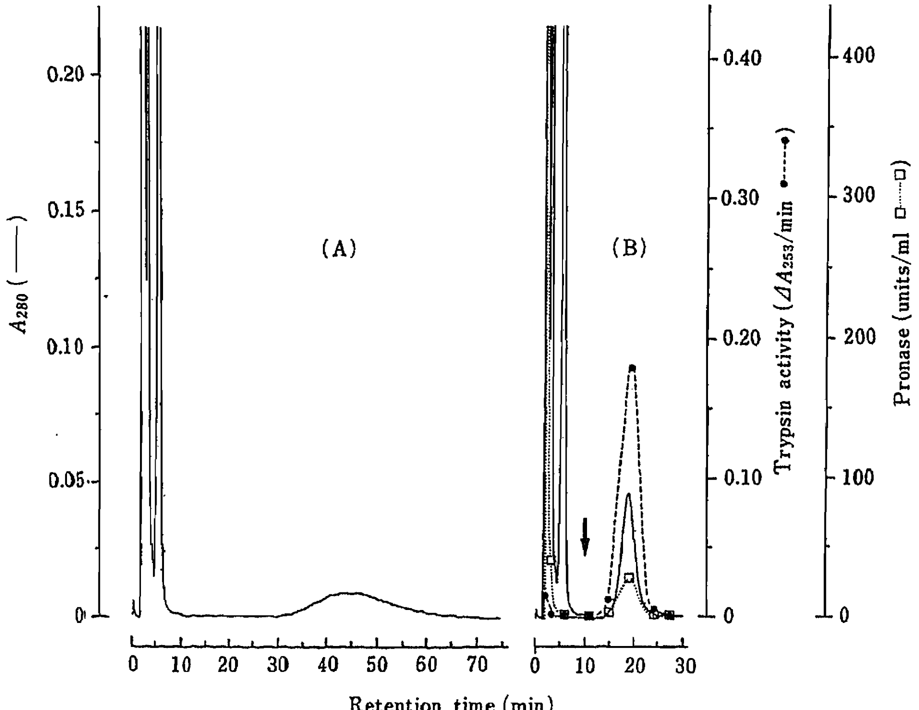

Fig. 2. HPLAC of *S. griseus* Trypsin on ABA-TSKgel G3000PW I

Pronase E solution (100 μl) in eluent 1 ($A_{280}=26$) was loaded on the column (7.5 mm i.d. × 7.5 cm) at a flow-rate of 1 ml/min at room temperature. The elution was performed isocratically in A. The eluent was changed at the point indicated by the arrow to eluent 1+20 mM AHA (pH 7.8) in B.

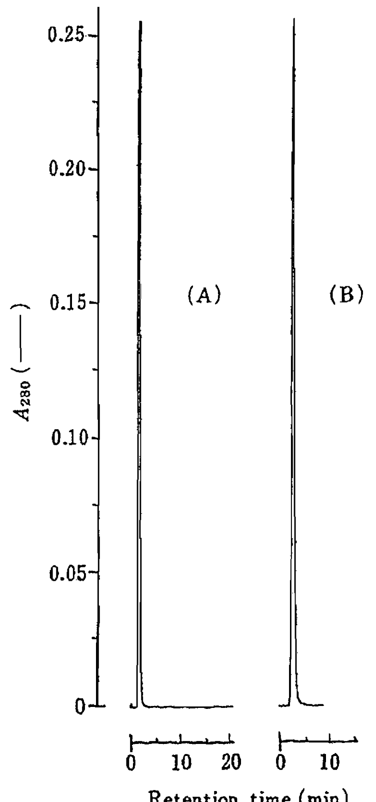

Fig. 3. HPLC of Chicken Lysozyme and Bovine Serum Albumin

Chromatographic conditions were as described in the legend to Fig. 1A except that 100 μl of chicken egg lysozyme solution ($A_{280}=1.7$) (A) or bovine serum albumin solution ($A_{280}=7.4$) (B) was loaded on the column.

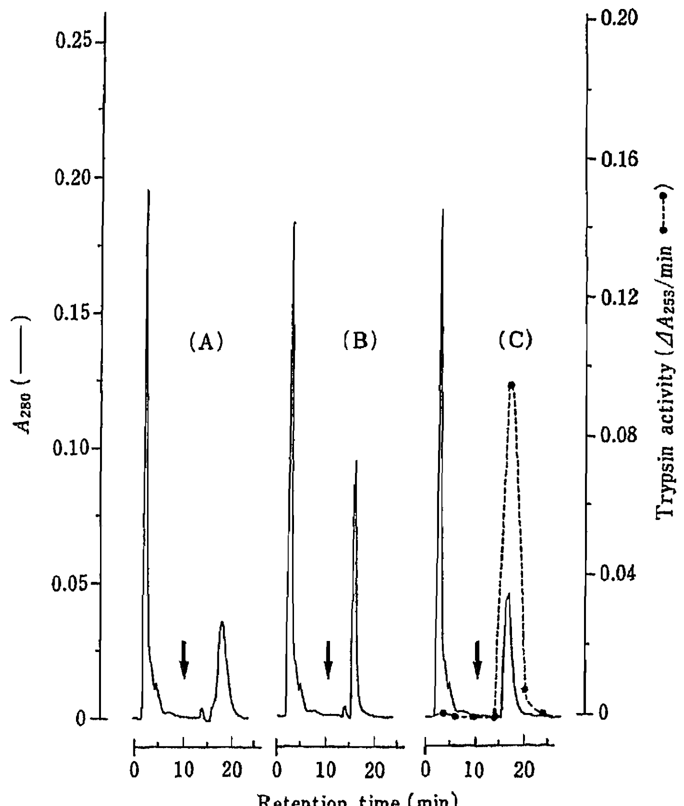

Fig. 4. HPLAC of Bovine Trypsin on ABA-TSKgel G3000PW II

Chromatographic conditions were as described in the legend to Fig. 1. The eluents were changed at the points indicated by the arrows to 0.1 M acetate buffer, pH 4.5 (eluent 2) in A, to eluent 2+20 mM AHA in B and to eluent 2+20 mM AHA+10 mM $CaCl_2$ in C.

concentration was 19 μmol/g wet gel, and the immobilization yield was as high as 98%.

Affinity Chromatography of Trypsin

Bovine trypsin or pronase E dissolved in eluent 1 was applied to columns of adsorbents. Pronase E is a mixture of proteinases, including *Streptomyces griseus* trypsin.[3] In the case of adsorbent I, the enzymes were eluted as retarded peaks at around 15 min for bovine trypsin (Fig. 1A) and at around 45 min for *S. griseus* trypsin (Fig. 2A). When 6-aminohexanoic acid (20 mM) was added to the eluting buffer,[7] the peaks of the enzymes became sharp and the retention times were shortened to 12 min for bovine trypsin (Fig. 1B) and 19 min for *S. griseus* trypsin (Fig. 2B). Proteins having no trypsin activities such as chicken egg lysozyme and bovine serum albumin passed through the column without retardation (Fig. 3) and the recovery of protein was 100% in both cases.

In the case of adsorbent II, trypsins were adsorbed on the column. Bovine trypsin was eluted with 0.1 M acetate buffer, pH 4.5 (Fig. 4A). The less the ionic strength of the eluting buffer was, the sharper the peak of the eluted enzyme was. Addition of AHA to the eluting buffer was also found to be effective to sharpen the trypsin peak (Fig. 4B). Since calcium ions prevent autolysis of trypsin,[8] which is pronounced at pH higher than 5, 10 mM $CaCl_2$ was added to the eluting buffer. In this case, bovine trypsin was eluted at 16 min (Fig. 4C). *S. griseus* trypsin was eluted with 0.2 M NaCl–HCl–20 mM AHA, pH 2.5, at 24 min (Fig. 5A). Lysozyme and serum albumin were not retained on the column, as was observed for adsorbent I.

On both chromatographies, the specific activities of bovine trypsin and *S. griseus* trypsin

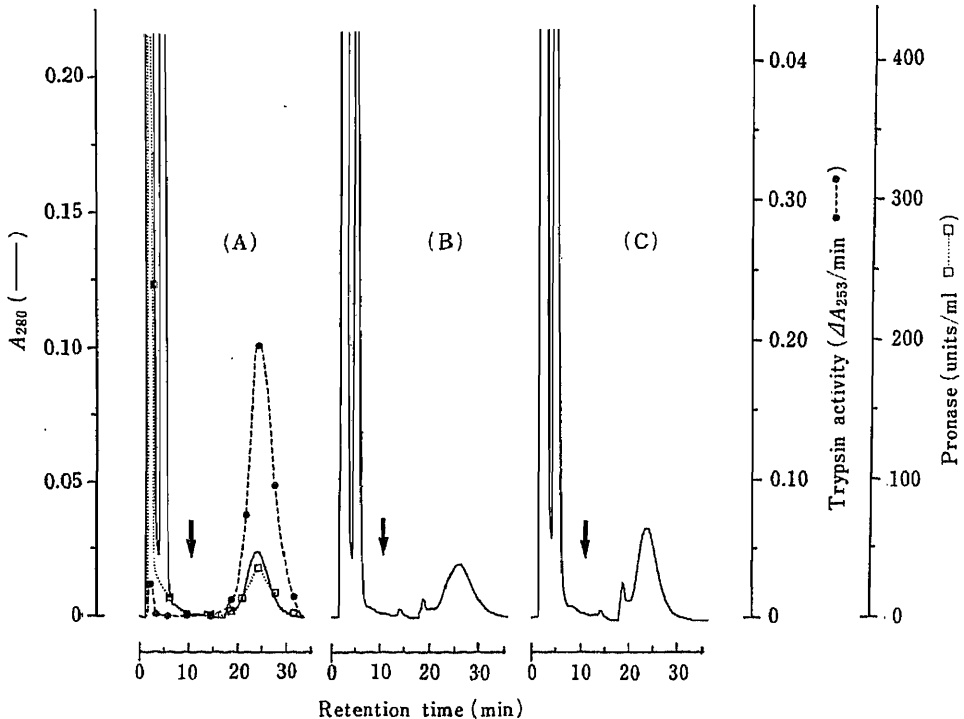

Fig. 5. HPLAC of *S. griseus* Trypsin on ABA-TSKgel G3000PW II

Chromatographic conditions were as described in the legend to Fig. 2. The eluent was changed at the points indicated by the arrow to 0.2 M NaCl–HCl–20 mM AHA, pH 2.5, and the effluent was mixed with eluent 1 immediately. Chromatographies were performed at 25 °C in A, 23 °C in B and 26 °C in C.

increased 3-fold and 16-fold per $A_{280\,\mathrm{nm}}$ unit, respectively. Pronase activity was found in the flow-through fractions, and its specific activity increased 1.7-fold on adsorbent I, and 1.3-fold on adsorbent II. The differences may be because the gel chromatographic separation of the first and second peak in the three flow-through peaks was better on adsorbent I than on adsorbent II. In all the chromatographies, the peaks of the enzymes became sharper when the temperature was elevated, as shown in Fig. 5. The small peaks just before the peaks of trypsins as seen in Fig. 4 or 5 arose from the changes of the eluting buffer.

Discussion

Two kinds of trypsin adsorbents for HPLAC were efficiently prepared by convenient procedures in this study. TSKgel G3000PW derivatives having carboxyl groups or glyceryl groups can be stored for a long time before use for immobilization of ligands. All the adsorbents have stable linkages between affinity ligands and carriers and can be stored for a long time. Adsorbent prepared with formyl carriers (adsorbent II) has no charged groups which might allow non-specific electrostatic adsorptions or disturb the specific interactions. Both adsorbents had practically no non specific adsorptions, *e.g.*, proteins having no trypsin activities such as lysozyme and bovine serum albumin were not retained on the columns (Fig. 3).

Ito *et al.* used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for the coupling of ABA to the carboxyl derivative of Asahipack GS-gel.[3] However, the coupling reaction using EDC requires strict control of pH and the coupling yield is not so high. In this study, we adopted EEDQ for this purpose. The coupling reaction using EEDQ proceeds over wide range of pH with very high yield.[9-11] However, the ligand concentration of adsorbent I thus

prepared could not be precisely determined by the methods we had employed. This was because not only ABA but also quinoline (produced from EEDQ upon activation of carboxyl groups[9]) has UV adsorbance at 320 nm and is reactive to 2,4,6,-trinitrobenzene sulfonate. Futhermore, we could not identify the optimal conditions for hydrolysis of the adsorbents to yield free ABA.

HPALC using ABA-TSKgel G3000PW columns was completed in a shorter time as compared with that using an ABA-Asahipak GS-gel column.[3] This may be because ABA-TSKgel G3000PW I and II have over tenfold higher ligand concentrations than ABA-Asahipak GS-gel.

With many affinity adsorbents, the introduction of a spacer between the ligand and carrier has resulted in successful affinity adsorption of various biological substances, *e.g.*, *Escherichia coli* β-galactosidase[12] and *Staphylococcus* nuclease.[13] Most trypsin adsorbents prepared with ABA also have long spacer arms.[1-3,14-16] However we have reported that long spacer arms are not necessary for adsorption of trypsin, and adsorbents having short spacers exhibited among the highest adsorption capacities for trypsin.[1] In this study, ABA immobilized with the long spacer (adsorbent I) was shown to have lower affinity for trypsin than ABA immobilized with the short spacer. This may be because the long spacer (having hydrophobic sites) interacts with hydrophobic portions of the carrier and does not function as a spacer arm, or immobilized ABA interacts electrostatically with carboxyl groups on the gel and does not function as a ligand.

When a column of 7.5 mm i.d. × 7.5 cm was used, trypsins were eluted in sharp retarded peaks on adsorbent I and were completely adsorbed on adsorbent II. The results indicate that adsorbent I is useful for rapid analysis of the enzymes and adsorbent II for preparative use. However, bovine trypsin was eluted in a sharp retarded peak with eluent 1 + 20 mM AHA on a short column (6 mm i.d. × 1 cm) of adsorbent II (data not shown), indicating that adsorbent II is also available for analytical use.

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Cellular Interaction and *in Vitro* Antitumor Activity of Lipophilic Mitomycin C Prodrugs

SATOSHI MATSUMOTO, YOSHIE HAYASHI, MITSURU HASHIDA, and HITOSHI SEZAKI*

Department of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Cellular interaction and *in vitro* antitumor activities of lipophilic prodrugs of mitomycin C (MMC) were studied in order to clarify their mode of action. Five lipophilic derivatives with various lipophilic promoieties (benzylcarbonyl, benzyloxycarbonyl, pentyloxycarbonyl, nonyloxycarbonyl, and cholesteryloxycarbonyl groups) were tested. All the derivatives except for cholesteryloxycarbonyl MMC were converted to MMC in the supernatant of tumor cell homogenate.

Lipophilic derivatives, especially nonyloxycarbonyl MMC and cholesteryloxycarbonyl MMC, associated with Ehrlich ascites carcinoma (EAC) cells more readily than MMC. While the association percentage remained almost constant during the course of incubation at 4 °C, it increased with incubation period at 37 °C, suggesting metabolic consumption of lipophilic derivatives in the tumor cells. Association percentages of derivatives at 4 °C were closely correlated to their partition coefficients between chloroform and water. The apparent distribution ratio of benzyloxycarbonyl MMC between EAC cells and the incubation medium also correlated with their volume ratio. These results suggested that lipophilic derivatives were incorporated into tumor cells through partition equilibrium between lipid components of tumor cells and the medium.

In vitro antitumor activities of lipophilic MMC derivatives were studied using EAC and L1210 leukemia cell culture. In the continuous exposure experiment, lipophilic derivatives which were converted to MMC in tumor cells showed equal or somewhat lower growth inhibitory activity as compared with MMC. In the case of 1 h or 5 min exposure, nonyloxycarbonyl MMC was more active than MMC, indicating that the growth-inhibitory effects of lipophilic derivatives are closely related to both cellular interaction and conversion rate to MMC.

Keywords——mitomycin C; lipophilic prodrug; L1210 leukemia; Ehrlich ascites carcinoma; cellular interaction; *in vitro* antitumor activity; cell culture system

Introduction

In cancer chemotherapy, it is desirable to deliver anticancer drugs selectively to the tumor site and to minimize the drug exposure of normal tissues in order to obtain high therapeutic efficacy.[1] In the pharmaceutical field, the prodrug approach is one of the most promising means of improving drug delivery, by altering the biopharmaceutical characteristics through the introduction of promoieties with suitable physicochemical properties.[2]

On the basis of this consideration, we have synthesized various kinds of prodrugs of mitomycin C (MMC) by coupling it with polymer beads,[3] polysaccharide,[4] and polyamino acid,[5] and examined the therapeutic characteristics of the products. In a series of investigations, some lipophilic MMC prodrugs were also developed by substituting the la-N-position of MMC with lipophilic carrier moieties.[6,7] These compounds showed significant *in vivo* antitumor activities when given by intraperitoneal injection and a stability study revealed that they were converted to MMC mostly by enzymatic hydrolysis.[8] Enhanced dermal absorbabilities, which resulted in improvement of the efficacy in topical use, were observed in

these compounds.[9] Chemical modification of a lipophilic prodrug was also shown to be a useful approach to improve the applicability of MMC to liposome and O/W emulsion formulations.[10-12] Combined delivery systems of lipophilic prodrugs with physical devices showed improved pharmacokinetic behavior such as sustained retention in the injection site and enhanced lymphatic transport after local injection, and significant anticancer activities against murine tumors were observed.[10,12] However, detailed information has not been reported about their mode of action at the cellular level.

In the previous papers, we dealt with the cellular interaction of polymeric prodrugs of MMC such as dextran conjugate and a polyamino acid conjugate in relation to their physicochemical properties.[13] In this investigation, the cellular interaction and *in vitro* antitumor activity of five lipophilic derivatives of MMC were studied in order to clarify their mode of action. The physicochemical characteristics of the derivatives are discussed from the viewpoint of prodrug design.

Experimental

Material——MMC was kindly supplied by Kyowa Hakko Kogyo Co. Five lipophilic derivatives of MMC (Table I) were synthesized as described previously.[6,7] Other chemicals were reagent-grade products obtained commercially.

Cellular Interaction of Lipophilic MMC Derivatives——Tumor cells were suspended in *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-buffered Hanks' balanced salt solution (10^7 cells/ml), and a drug solution in ethanol (160 μg eq MMC/ml) was added to give a final concentration of 10 μg eq MMC/ml. The mixture incubated for a definite time at 37 or 4 °C. After centrifugation at 3000 rpm for 5 min, the drug concentration in the supernatant was measured spectroscopically and the association percentage was calculated. The effects of temperature, drug concentration and cell density on cellular interaction were examined.

***In Vitro* Antitumor Activity with Continuous Drug Exposure**——Murine L1210 leukemia or Ehrlich ascites carcinoma (EAC) cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Company, NY), then the drug solution in ethanol was added, and the mixture was seeded on a multiwell tissue culture plate (Becton, Dickinson and Co., CA) at a density of 10^5 cells/ml/well. The final concentration of ethanol was less than 1% and this concentration did not affect cell growth. After incubation in a humidified atmosphere containing 5% CO_2 at 37 °C for 72 h, viable cells were counted with a Bürker–Turk hemocytometer by the trypan blue exclusion method. The growth inhibitory effect was assessed as follows: growth inhibition (%) $= (1 - T/C) \times 100$, where T and C represent the number of surviving cells in a treated group and that in an untreated control group, respectively. Experiments were carried out in triplicate.

***In Vitro* Antitumor Activity with 1 h or 5 min Drug Exposure**——Tumor cells were exposed to various concentrations of drugs in Hanks' solution for 1 h or 5 min at 37 °C and then were washed twice with the same medium by centrifugation, as described previously.[13] Drug-treated cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated for 4 d. Growth inhibition was determined as above.

Conversion of Lipophilic MMC Derivatives to MMC——EAC cells (10^8 cells/ml) were homogenized in HEPES-buffered Hanks' solution (pH 7.2) with a glass-Teflon homogenizer under ice-cooling. The homogenate was centrifuged at 3000 rpm for 5 min. MMC derivatives were added to the supernatant of the cell homogenate at a final concentration of 30 μg eq MMC/ml and incubated at 37 °C. The incubation was stopped by addition of ice-cold acetonitrile (4 volumes with respect to the sample). After being shaken for 10 min, the mixture was filtered through a Micro Filter FR-20 (Fuji Photo Film Co.). Derivatives and regenerated MMC in the filtrate were determined by high performance liquid chromatography (system LC-5A, Shimadzu) with a variable-wavelength UV detector (SPD-2A, Shimadzu) and a Chromatopack CR2AX (Shimadzu) in a reverse-phase mode. The stationary phase used was Cosmosil 5C18 (Nakarai Chemicals) and a short column packed with RP-2 (E. Merck, Germany) was used to guard the main column. Methanol–water was used as the mobile phase at a flow rate of 0.8 ml/min. Standard solutions were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

Results

Conversion of Lipophilic MMC Derivatives to MMC

Table I summarizes the structures and physicochemical characteristics of lipophilic MMC derivatives. All the derivatives were degraded conforming to first-order kinetics in the supernatant of EAC cell homogenate. Under the condition used, the conversion of cholester-

Table I. Structures and Physicochemical Characteristics of Lipophilic Mitomycin C Derivatives

Compound	R	PC[a]	Degradation rate constant (h^{-1})	
			EAC	Rat liver[b]
MMC	-H	0.259	0.046	
Benzylcarbonyl MMC	$-COCH_2-C_6H_5$	163	0.036[c]	0.841[c]
Benzyloxycarbonyl MMC	$-COOCH_2-C_6H_5$	1643	0.050[c]	25.61[c]
Pentyloxycarbonyl MMC	$-COO-C_5H_{11}$	1785	0.015[c]	4.35[c]
Nonyloxycarbonyl MMC	$-COO-C_9H_{19}$	29195	0.036[c]	2.62[c]
Cholesteryloxycarbonyl MMC	$-COO-C_{27}H_{45}$	8216	0.015	0.073

a) Partition coefficient between chloroform and water (from ref. 11). *b*) Values are cited from refs. 7) and 9). *c*) MMC was regenerated during the incubation period.

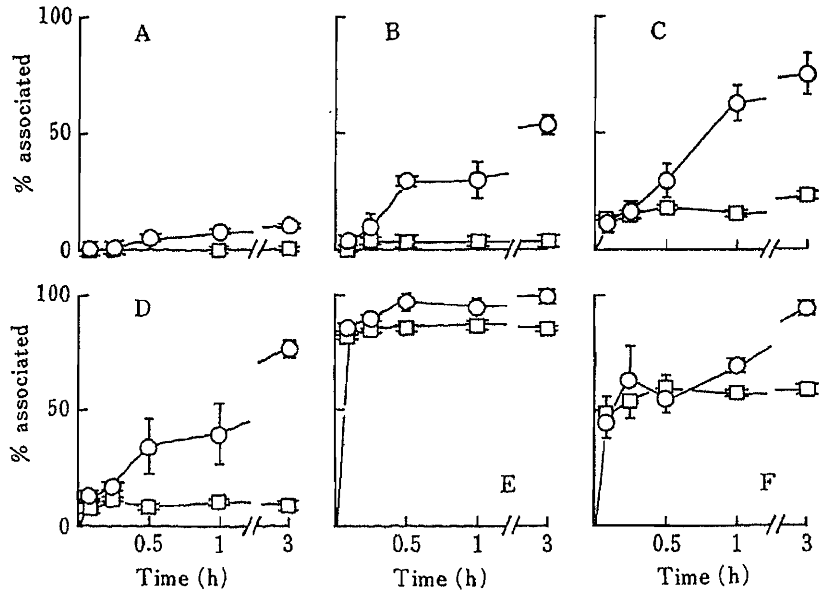

Fig. 1. Uptake Time Courses of MMC and Lipophilic MMC Prodrugs by Ehrlich Ascites Carcinoma Cells at 37 °C or 4 °C

A), MMC; B), benzylcarbonyl MMC; C), benzyloxycarbonyl MMC; D), pentyloxycarbonyl MMC; E), nonyloxycarbonyl MMC; F), cholesteryloxycarbonyl MMC.
○, 37 °C; □, 4 °C.
Ehrlich ascites carcinoma cells were incubated in Hanks' balanced salt solution containing MMC or a lipophilic MMC prodrug at 37 or 4 °C. After incubation for the indicated periods of time, cells were separated by centrifugation and the drug concentration in the supernatant was determined spectroscopically. Association percentage was calculated and expressed as the mean ± S.D.

yloxycarbonyl MMC into MMC was not observed, but all other derivatives were converted to MMC, as they are in the liver homogenate or plasma of rodents.[7,9] The conversion rates in EAC cell homogenate were slower than those in the liver homogenate or plasma.

Cellular Interaction of Lipophilic MMC Derivatives

Figure 1 shows association–time profiles of lipophilic MMC derivatives with EAC cells. At 4 °C, lipophilic derivatives, especially nonyloxycarbonyl and cholesteryloxycarbonyl MMC, associated with EAC cells more efficiently than MMC. Association percentages of drugs remained almost constant during the incubation period. On the other hand, association of lipophilic derivatives at 37 °C increased with increasing incubation period and was higher than that at 4 °C.

Figure 2 illustrates the relation between association percentage of lipophilic derivatives

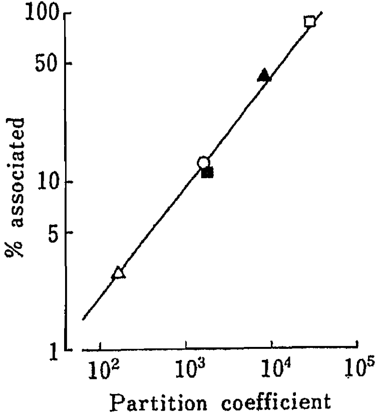

Fig. 2. Relationship between Partition Coefficient and Association Percentage with Ehrlich Ascites Carcinoma Cells of Lipophilic Prodrugs

Abscissa; partition coefficient between chloroform and water cited from ref. 11. Ordinate; association percentage obtained from Fig. 1 (4 °C, 15 min).

△, benzylcarbonyl MMC; ○, benzyloxycarbonyl MMC; ■, benzyloxycarbonyl MMC; □, nonyloxycarbonyl MMC; ▲, cholesteryloxycarbonyl MMC.

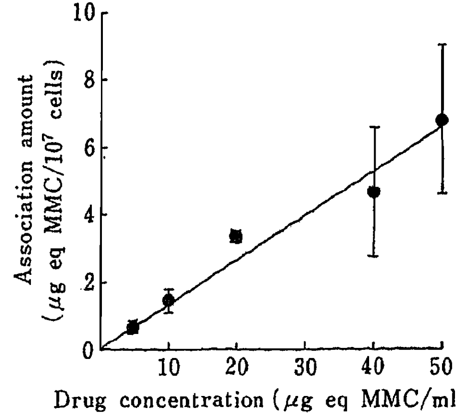

Fig. 3. Effects of Concentration of Benzyloxycarbonyl MMC on Its Association with Ehrlich Ascites Carcinoma Cells in 15 min at 4 °C

Uptake amount was determined as described in the legend to Fig. 1. The results are expressed as the mean ± S.D.

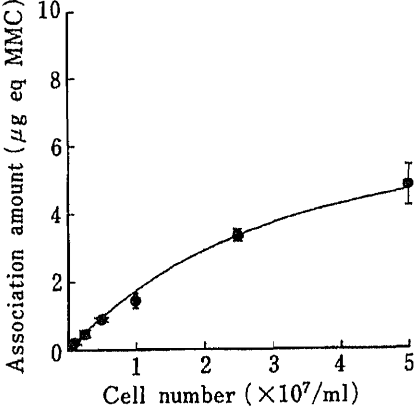

Fig. 4. Effect of Number of Ehrlich Ascites Carcinoma Cells on the Cellular Association of Benzyloxycarbonyl MMC

Uptake amount was determined as described in the legend to Fig. 1. The results are expressed as the mean ± S.D.

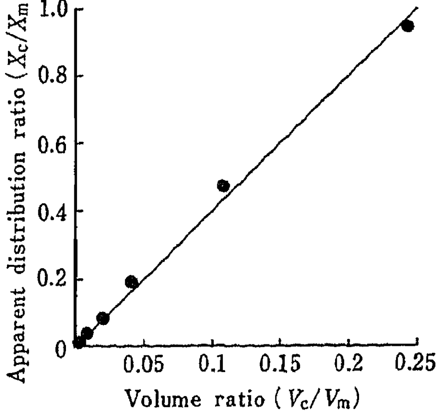

Fig. 5. Relationship between the Ratio of Drug Amount Associated with EAC Cells (X_c) to That in the Medium (X_m) and the Ratio of Cellular Volume (V_c) to Medium Volume (V_m)

Drug amount associated with EAC cells and that in the medium after incubation for 15 min at 4 °C were determined as described in the legend to Fig. 1. Cellular volume was calculated by assuming the cell to be a sphere with a diameter of 20 μm.

at 4 °C and their partition coefficients between chloroform and water; there is a linear relationship.

In order to characterize the cellular interaction in detail, association experiments were carried out under various conditions employing benzyloxycarbonyl MMC as a model compound. Figure 3 shows the effect of concentration of benzyloxycarbonyl MMC on the cellular association at 4 °C. Association amount increased in proportion to drug concentration in the medium.

Figure 4 shows the effect of cell number on total drug association at 4 °C. The amount of associated drug increased with increase in cell number. Based on the results shown in Fig. 4, the apparent distribution ratio of benzyloxycarbonyl MMC between the EAC cells and the incubation medium was calculated. A plot of apparent distribution ratio against the ratio of cell volume to medium volume gave a straight line (Fig. 5) which is consistent with the following equation;

$$X_c/X_m=(C_cV_c)/(C_mV_m)=(C_c/C_m)(V_c/V_m)=P_c(V_c/V_m)$$

where X_c and X_m represent drug amount incorporated in the tumor cells and that remaining in the incubation medium, C_c and C_m represent drug concentration in the cells and medium, and V_c and V_m represent cellular volume and medium volume, respectively. P_c represents a partition parameter between the cells and medium and was calculated to be about 4 under the conditions used.

In Vitro Antitumor Activity of Lipophilic MMC Derivatives

Figure 6A, B, and C shows the growth inhibitory effects of MMC derivatives on L1210 cells in continuous, 1 h, and 5 min exposure experiments, respectively. MMC showed growth-inhibitory effects at the lowest concentration in the continuous exposure experiment. Nonyloxycarbonyl MMC and benzylcarbonyl MMC exhibited essentially equal activity to

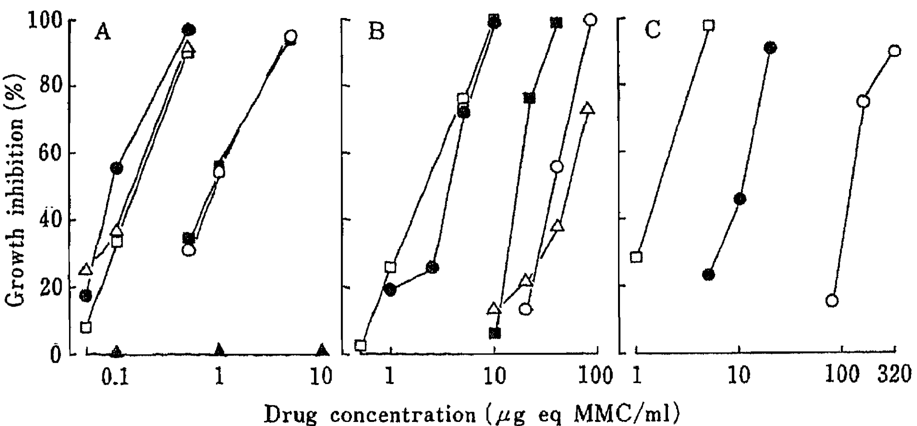

Fig. 6. Growth-Inhibitory Effects of MMC and Lipophilic MMC Prodrugs on L1210 Leukemia Cells

●, MMC; △, benzylcarbonyl MMC; ○, benzyloxycarbonyl MMC; ■, pentyloxycarbonyl MMC; □, nonyloxycarbonyl MMC; ▲, cholesteryloxycarbonyl MMC.

A) L1210 leukemia cells were cultured for 72 h in RPMI 1640 medium supplemented with 10% fetal bovine serum and various concentrations of MMC or a lipophilic MMC prodrug. Viable cells were counted by the trypan blue exclusion method and the growth inhibition percentage was calculated.

B) L1210 leukemia cells were exposed to various concentrations of MMC or a lipophilic MMC prodrug for 1 h at 37 °C in Hanks' balanced salt solution. After being washed twice with the same medium, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum for 4 d. Growth inhibition percentage was determined as above.

C) Growth inhibition was determined as described in the legend to Fig. 6B) except that cells were exposed to drugs for 5 min.

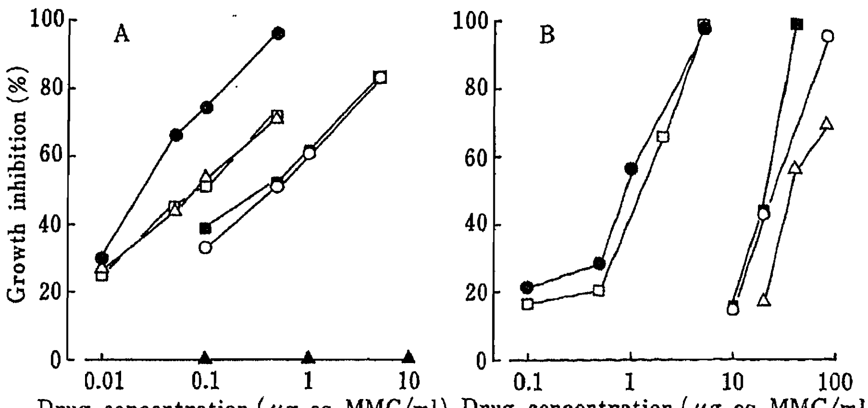

Fig. 7. Growth-Inhibitory Effects of MMC and Lipophilic MMC Prodrugs on Ehrlich Ascites Carcinoma Cells

●, MMC; △, benzylcarbonyl MMC; ○, benzyloxycarbonyl MMC; ■, pentyloxycarbonyl MMC; □, nonyloxycarbonyl MMC; ▲, cholesteryloxycarbonyl MMC.
A) Growth inhibition was determined as described in the legend to Fig. 6A).
B) Growth inhibition was determined as described in the legend to Fig. 6B).

MMC but other derivatives were somewhat less active. Cholesteryloxycarbonyl MMC, which was not converted to MMC, failed to inhibit cell growth. In the 1 h exposure experiments, nonyloxycarbonyl MMC, which interacted strongly with tumor cells, showed higher activity than MMC. Other derivatives exhibited lower activity. In the 5 min exposure experiment, the growth-inhibitory activity of nonyloxycarbonyl MMC was much higher than that of MMC.

Figure 7 shows growth inhibitory effects of MMC derivatives on EAC cells during continuous exposure and 1 h exposure. Derivatives showed similar activities against EAC cells in each exposure experiment.

Discussion

Among various physicochemical parameters, molecular lipophilicity plays a dominant role in determining the biopharmaceutical characteristics of drugs.[14] Many attempts have been made to avoid enzymatic degradation,[15] enhance the uptake by tumor cells,[16] improve the encapsulation efficiency into liposomes,[17] and accelerate intestinal absorption by controlling the lipophilicity of the drug.[18-20]

In previous studies, we developed lipophilic prodrugs of MMC, an amphiphilic anticancer drug,[6,7] aiming at the improvement of its pharmacokinetic behavior and applicability to lipoidal dosage forms. We subsequently elucidated their pharmaceutical and pharmacological characteristics.[8-10] In this paper, the cellular interaction and growth-inhibitory activity of lipophilic MMC derivatives were examined and compared with those of other types of MMC prodrugs.

Lipophilic MMC derivatives showed greater interaction with EAC cells both at 4 and 37 °C, and a good correlation was observed between the association percentage and partition coefficient in chloroform/water of these prodrugs. Cellular association at 37 °C increased with incubation time except in the case of nonyloxycarbonyl MMC, while the uptake percentages at 4 °C remained almost constant. These results suggested that the derivatives were metabolically consumed in the tumor cells at 37 °C. In the case of nonyloxycarbonyl MMC, which is also metabolically converted to MMC, the increase in association amount was not significant since 85% of the applied dose was already incorporated into tumor cells at the initial period of incubation. Cellular interaction of lipophilic derivatives with other cell lines including L1210 leukemia, P388 leukemia, and AH 66 ascites hepatoma was essentially similar to that with

EAC cells (data not shown). Therefore, we used EAC cells, which are easily obtainable from ascitic fluid of mice, for further examination of cellular interaction.

As shown in Fig. 3, the uptake of benzyloxycarbonyl MMC by EAC cells at 4 °C was proportional to its concentration in the medium. The association of benzylcarbonyl MMC also increased with increase of cell number (Fig. 4). The apparent distribution ratio between EAC cells and the incubation medium correlated well with the volume ratio (Fig. 5). These findings suggest that lipophilic derivatives are incorporated into tumor cells by partitioning to cellular lipid components. The possibility of enhancing cellular access by increasing lipophilicity through the prodrug approach is clear from these results.

MMC has been shown to cross-link double-helical deoxyribonucleic acid (DNA) after enzymatic reduction to the corresponding hydroquinone.[21] These processes are accelerated under hypoxic conditions and result in selective toxicity to chronically hypoxic tumor cells[22,23] Positions 1a and 10 appear to be the alkylating sites of MMC, and their alkylating ability is enhanced when methanol is eliminated from the produced hydroquinone to give the indolehydroquinone.[24] Consequently it is considered that substitution at the 1a position leads to diminution of the biological activity.[6,7] Lipophilic derivatives showed slightly lower cytotoxicities than MMC in the continuous exposure experiments (Figs. 6A and 7A). Cholesteryloxycarbonyl MMC was not converted to MMC even in the biological media (Table I) and did not show any cytotoxic activity. These results indicate that lipophilic MMC derivatives have to be converted to MMC in order to exhibit cytocidal effect. Thus, the lability of prodrugs should play an important role in the manifestation of their antitumor activities. The linkage structure between MMC and the lipophilic promoiety is the determinant of this property. [8]

As reported previously, plasma and liver homogenate of rats or mice successfully catalyzed the hydrolysis of MMC derivatives having the carbamate linkage, except for cholesteryloxycarbonyl MMC.[7] Nonspecific esterase or carbamidase might be responsible for this reaction. These compounds were also converted to MMC by EAC cell homogenate, as shown in Table I. In the 1 h or 5 min exposure experiment, nonyloxycarbonyl MMC, which strongly interacted with tumor cells, exhibited higher cytocidal activity than MMC, but other derivatives showed less activity. These results suggested that cellular interaction is also an important factor determining the cytotoxic activity. Extremely high lipophilicity (partition coefficient $> 10^4$) seems to guarantee sufficient retention of the active species in the tumor cell even after repeated washing. Similar considerations might apply *in vivo* when the drug exposure is limited to a short period.

Lipophilic prodrugs must satisfy the following two criteria to exhibit potent activities: 1) high cellular association, 2) full conversion to the parent drug at an adequate rate. Lipophilic modification of an antitumor drug renders the drug more able to permeate into cells, and therefore such modification is a promising approach for further development of anticancer drugs. It might also be possible to overcome the drug resistance of tumor cells in which drug uptake is impaired.

Another possible approach to improve the pharmacokinetical properties and cellular access of MMC is conjugation to a high-molecular-weight compound. We have developed macromolecular derivatives of MMC, MMC–dextran conjugate (MMC-D)[4] and MMC–polyamino acid conjugates,[5] and investigated their physicochemical,[25,26] biopharmaceutical,[27,28] and chemotherapeutic characteristics.[29] These macromolecular prodrugs of MMC were designed to liberate MMC by chemical hydrolysis in contrast with lipophilic MMC prodrugs.[1] MMC-D with cationic charge and MMC–polylysine conjugate were strongly adsorbed on tumor cell surface through electrostatic interaction.[5,13] Adsorption of MMC-D increased with increase of its molecular weight and conformed to Langmuir's adsorption isotherm. A good correlation was observed between the growth-inhibitory effects of MMC-D

and the extent of its cellular interaction, and cationic MMC-D was more active than MMC in the 1 h exposure experiment. As with the lipophilic prodrugs of MMC demonstrated in this study, the cellular interaction and release rate of MMC were concluded to play an important role in the manifestation of the antitumor activity of macromolecular MMC prodrugs.

Improvement of the access of antitumor agents to target tumor cells is one of the most important aims in designing prodrugs. We have applied two approaches to one conventional drug, MMC, with considerable therapeutic success. In these studies, lipid components and anionic surface materials of the tumor cell were chosen as the target and lipophilic small molecules and cationic macromolecules were tested as carrier moieties of the prodrug. The close relationship of lipophilicity and electrostatic properties of prodrugs with their cellular interactions was explored from the viewpoints of physicochemistry. The significance of regeneration process also had been elucidated and the guide for designing linkage structures was given for each approach. Through these reports, possibility and a rational avenue for improving cellular accessibility of antitumor agent by prodrug design have been demonstrated.

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Cellular Interaction and *in Vitro* Antitumor Effect of Various Mitomycin C Prodrugs in Mitomycin C-Resistant L1210 Leukemia Cell Lines

SATOSHI MATSUMOTO, TSUNEHIKO SHIGEOKA, YOSHINOBU TAKAKURA, MITSURU HASHIDA, and HITOSHI SEZAKI*

Department of Basic Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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L1210 mouse leukemia cell lines resistant to mitomycin C (MMC) were established by repeated continuous exposure of parental L1210 leukemia cells to MMC *in vitro*. The established cell lines showed 13-fold or more resistance to MMC compared with the parental cell line. A macromolecular prodrug of MMC, MMC–dextran conjugate with cationic charge (MMC-$D_{cat.}$), and lipophilic MMC prodrugs were assessed for ability to circumvent the resistance of tumor cells against MMC. Association of MMC and its prodrugs with resistant cell lines was similar to that with the parental cell line. Higher cellular association of drugs was observed with increase of the molecular weight of MMC-D and with increase of the partition coefficient of lipophilic MMC prodrugs.

In vitro antitumor activities of MMC prodrugs against resistant cell lines were evaluated by determining cell number after cultivation for 3 or 4 d with the agents. In the continuous exposure experiment, the degrees of resistance to most prodrugs were almost the same as that to MMC. On the other hand, MMC-$D_{cat.}$ showed higher activity against parental L1210 cells but lower activity against resistant cells than MMC in the 1 h exposure experiment. In the case of lipophilic MMC prodrugs, nonyloxycarbonyl MMC showed higher activity against both parental and resistant cells compared with MMC. The degree of resistance to nonyloxycarbonyl MMC was one-fifth of that to MMC. Thus, it may be possible to attack the drug-resistance problem by means of the prodrug approach.

Keywords——mitomycin C; macromolecular prodrug; lipophilic prodrug; L1210 leukemia; mitomycin C-resistant cell line; cellular interaction; *in vitro* antitumor activity; cell culture system

Introduction

Mitomycin C (MMC) is a highly active antitumor antibiotic in both animal tumor systems and a variety of human neoplastic diseases. Its utility in human therapy, however, has been limited primarily by severe cumulative myelosupression or gastrointestinal damage.[1] These disadvantages could be overcome and the therapeutic efficacy enhanced if the cytotoxicity were localized at the tumor site and if the burden on other tissues could be minimized by improving the pharmacokinetic properties and cellular access of MMC.[2] One possible approach is the chemical transformation of the drug molecule into a latent from (prodrug).[3]

We have developed various kinds of derivatives of MMC by coupling it with polymer beads,[4] polysaccharide,[5] and polyamino acid,[6] or by substituting 1a-N-position with lipophilic carrier groups.[7,8] We have already reported their physicochemical,[9,10] pharmacological,[11,12] and pharmacokinetic characteristics.[13-15] Cellular interaction characteristics and cytotoxicity of these prodrugs have been examined in parental L1210 leukemia and Ehrlich ascites carcinoma cells, in relation to the mode of action.[16,17] Through these

investigations, some prodrugs of MMC were proved to have excellent therapeutic efficacy due to improved pharmacokinetic properties.

Development of drug resistance in malignant cells during treatment is another major obstacle to cancer chemotherapy.[18] From the clinical viewpoint, the absolute sensitivity of tumor cells to an anticancer drug, *i.e.*, the drug concentration required to kill tumor cells, is more important than the relative resistance compared with the parental cell line. Therefore, we have established MMC-resistant L1210 leukemia cell lines by continuous exposure of the parental L1210 cell line to MMC *in vitro*. We investigated the association of macromolecular prodrugs of MMC, cationic MMC–dextran conjugate (MMC-$D_{cat.}$) with molecular weights of 10000, 70000, and 500000, and lipophilic MMC prodrugs having a benzylcarbonyl, benzyloxycarbonyl, or nonyloxycarbonyl group, with the resistant cells. Their growth-inhibitory effects on MMC-resistant cells were compared with those on the parental MMC-sensitive cells. The possibility of overcoming drug resistance of tumor cells by means of the prodrug approach is discussed.

Experimental

Materials——MMC was kindly supplied by Kyowa Hakko Kogyo Co. Cationic MMC-D with a molecular weight of 10000 (T-10), 70000 (T-70) or 500000 (T-500), and lipophilic MMC derivatives coupled with benzylcarbonyl, benzyloxycarbonyl or nonyloxycarbonyl groups were synthesized as described previously.[5,7,8] All other chemicals were of reagent grade and were obtained commercially.

Cell Culture——The growth medium for parental L1210 leukemia cell line and its MMC-resistant sublines was RPMI 1640 medium supplemented with 10% fetal bovine serum (Grand Island Biological Co., NY). The cells were maintained in the growth medium in tissue culture dishes (Becton, Dickinson and Co., CA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Establishment of L1210 Cell Lines Resistant to MMC——Initial induction of resistance was achieved by continuous exposure of L1210 cells to a sublethal concentration of MMC (0.07 μg/ml) over 2 months. Medium change was carried out every 4—5 d. The concentration of MMC was gradually increased every 10 or more passages. During the culture, the cells were continuously exposed to MMC. After at least 10 passages with a certain concentration of MMC, cells were maintained in the growth medium without MMC. The degree of resistance, which was calculated as the ratio of IC_{50} (drug concentration giving 50% growth inhibition) of resistant cells to that of the parental cells, did not change over 6 months under these conditions. Obtained cell lines were named according to the final concentration of MMC; *i.e.*, cell line M1 was established by cultivation with a final MMC concentration of 1 μg/ml.

Cellular Interaction of MMC Prodrugs——Tumor cells were suspended in Hanks' balanced salt solution (10^6 cells/ml), concentrated drug solution was added to give a final concentration of 10 μg equivalent MMC/ml, and the mixture was incubated for a definite period at 37 °C. After centrifugation at 3000 rpm for 5 min, the drug concentration in the supernatant was measured spectroscopically and the association percentage was calculated.

Growth Inhibitory Effects of MMC Prodrugs with Continuous Exposure——Parental or MMC-resistant L1210 leukemia cells were suspended in the growth medium, drug solution was added, and the mixture was seeded on a multiwell tissue culture plate (Becton, Dickinson and Co., CA) at a density of 10^5 cell/ml/well. After incubation in a humidified atmosphere containing 5% CO_2 at 37 °C for 72 h, viable cells were counted with a Bürker–Turk hemocytometer by the trypan blue exclusion method. The growth inhibition was calculted as follows: growth inhibition (%) $= (1 - T/C) \times 100$, where T and C represent the number of surviving cells in a treated group and that in an untreated control group, respectively. Experiments were carried out in triplicate.

Growth Inhibitory Effects of MMC Prodrugs with 1 h Exposure——Cells were exposed to various concentrations of drugs in Hanks' balanced salt solution buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid for 1 h at 37 °C. Drug-treated cells were washed twice with the same medium, resuspended in the growth medium, and cultured for 96 h, and growth inhibition was determined as above.

Results

Characteristics of MMC Resistant L1210 Leukemia Cell Lines

The doubling times and drug sensitivities of MMC-resistant cell lines are summarized in Table I. The doubling time of the parental cell line was about 11 h and those of the resistant

cell lines were slightly longer. The degree of resistance was calculated as the ratio of IC_{50} for a resistant cell line to that for the parental cell line. A resistant cell line M1, which was finally exposed to 1 μg/ml of MMC, was approximately 14-fold more resistant to MMC as compared with the parental cell line. The extent of resistance to MMC increased with increase of the MMC concentration to which the cells were exposed during cultivation. MMC resistant cells also showed resistance to adriamycin, but the degree of resistance to adriamycin was smaller than that to MMC. The size and shape of the parental and resistant cells were almost the same as determined by microscopical observation. The cell lines which showed higher resistance were used for further study.

Cellular Interaction of MMC Prodrugs with MMC Resistant L1210 Leukemia Cells

Figures 1 and 2 show cellular association–time profiles of MMC-$D_{cat.}$ and lipophilic

Table I. Characteristics of MMC-Resistant L1210 Leukemia Cell Lines

Cell lines	Doubling time (h)	Mitomycin C		Adriamycin	
		IC_{50}[a] (μg/ml)	Degree of resistance[b]	IC_{50} (μg/ml)	Degree of resistance
Parental L1210	11.8	0.091	1	0.057	1
L1210 (M0.1)	16.0	0.165	1.81	N.D.[c]	
L1210 (M0.5)	12.3	0.780	8.57	N.D.	
L1210 (M1)	14.5	1.24	13.7	0.628	11.1
L1210 (M1.5)	17.5	1.38	15.2	N.D.	

a) Drug concentration showing 50% growth inhibition in the continuous exposure experiment. *b*) Calculated as the ratio of IC_{50} of MMC-resistant cells to that of parental cells. *c*) Not determined.

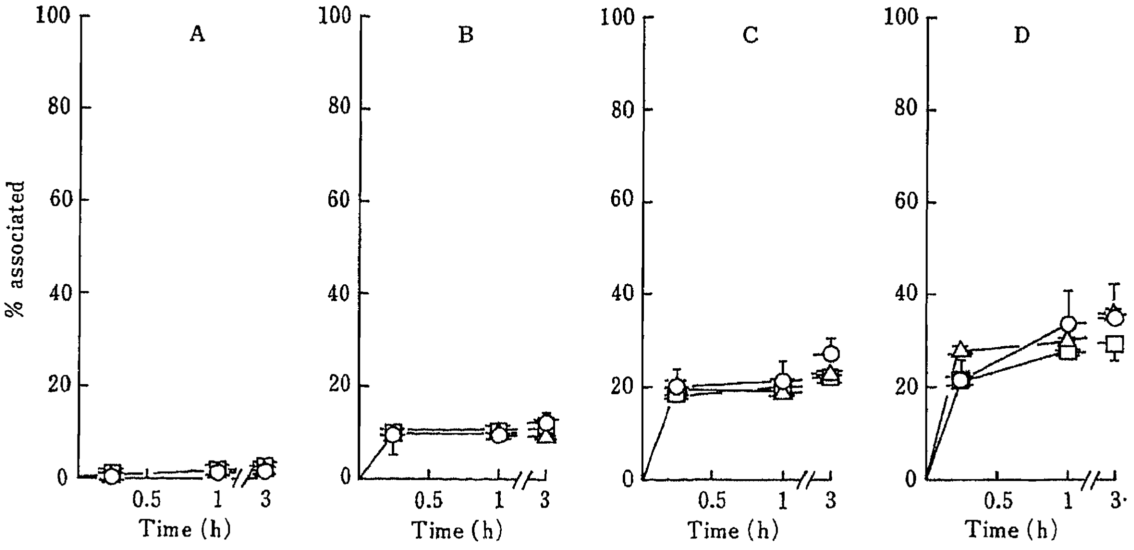

Fig. 1. Association Time Courses of Cationic MMC-D with Parental and MMC-Resistant L1210 Leukemia Cell Lines at 37 °C

A), MMC; B), MMC-$D_{cat.}$ (T-10); C), MMC-$D_{cat.}$ (T-70); D), MMC-$D_{cat.}$ (T-500).
○, parental L1210; □, L1210 (M1); △, L1210 (M1.5). Cells were incubated in Hanks' balanced salt solution containing MMC or cationic MMC-D at 37 °C. After incubation for the indicated periods of time, cells were separated by centrifugation and the drug concentration in the supernatant was determined spectroscopically. Results are expressed as the mean ± S.D.

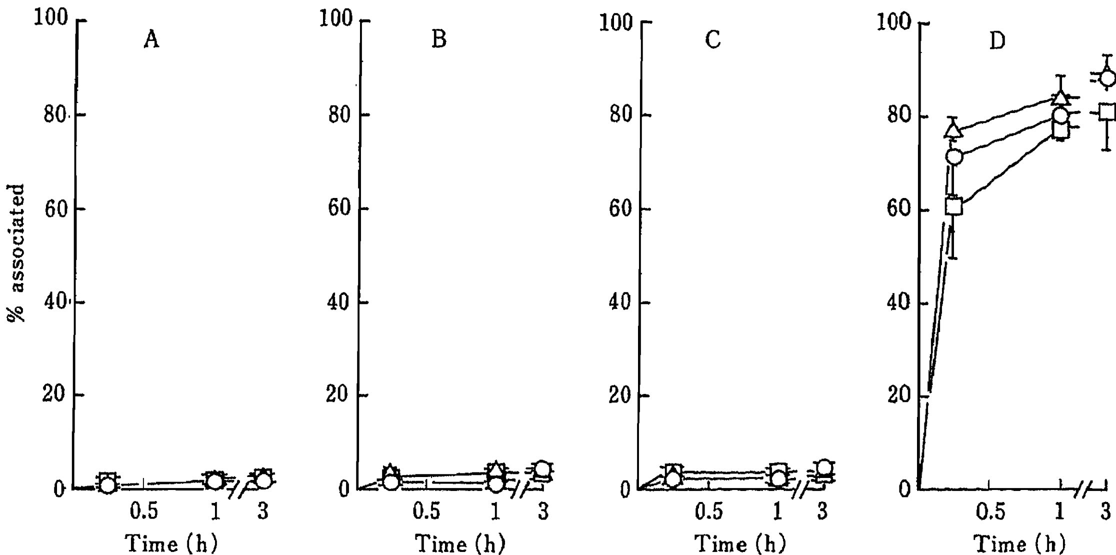

Fig. 2. Association Time Courses of Lipophilic MMC Prodrugs with Parental and MMC-Resistant L1210 Leukemia Cell Lines at 37 °C

A), MMC; B), benzylcarbonyl MMC; C), benzyloxycarbonyl MMC; D), nonyloxycarbonyl MMC.

○, parental L1210; □, L1210 (M1); △, L1210 (M1.5). Association percentages were determined as described in the legend to Fig. 1. Results are expressed as the mean ± S.D.

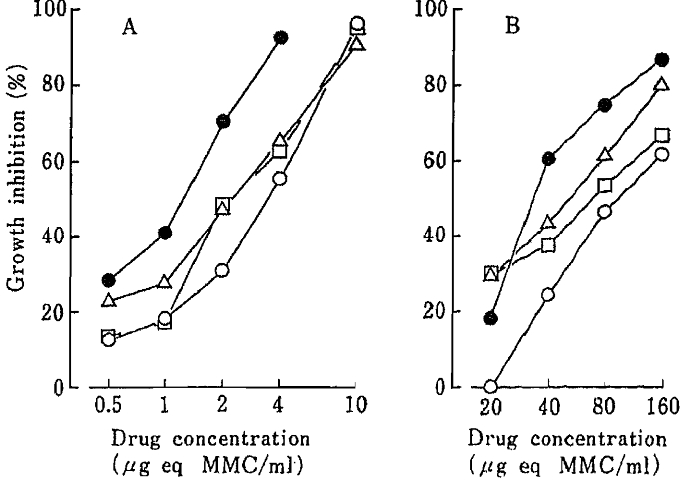

Fig. 3. Growth-Inhibitory Effects of Cationic MMC-D on MMC-Resistant L1210 Leukemia Cell Line (M1) in the Continuous Drug Exposure (A) or 1-h Drug Exposure (B) Experiment

●, MMC; ○, MMC-$D_{cat.}$ (T-10); □, MMC-$D_{cat.}$ (T-70); △, MMC-$D_{cat.}$ (T-500). Abscissa, logarithmic scale.

A) Cells were cultured for 72 h in RPMI 1640 medium supplemented with 10% fetal bovine serum and various concentrations of MMC or MMC-$D_{cat.}$. Viable cells were counted by the trypan blue exclusion method and growth inhibition percentage was calculated.

B) Cells were exposed to various concentrations of MMC or MMC-$D_{cat.}$ for 1 h in Hanks' balanced salt solution. After being washed twice with the same medium, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum for 4 d. Growth inhibition percentage was determined as above.

MMC prodrugs, respectively. There were relatively small differences between the association of all tested drugs with MMC-resistant cells and that with the parental cells. MMC prodrugs interacted strongly with both parental and resistant cells as compared with MMC. Greater

interaction was observed with an increase of molecular weight of dextran in the case of macromolecular prodrugs (Fig. 1). Nonyloxycarbonyl MMC, which has a highest partition coefficient in the chloroform/water system,[19] showed the highest uptake among the tested lipophilic prodrugs (Fig. 2). No significant difference was observed between uptake percentages of MMC prodrugs by the parental cell line and those by the resistant cell lines.

Growth-Inhibitory Effects of MMC Prodrugs

Figures 3 and 4 illustrate the growth-inhibitory effects of MMC-$D_{cat.}$ and lipophilic MMC prodrugs, respectively, on an MMC-resistant L1210 cell line (M1). In the continuous exposure experiment (Figs. 3A and 4A), prodrugs except for nonyloxycarbonyl MMC showed slightly lower activity than MMC against MMC-resistant cells.

In the case of the 1 h exposure experiment (Figs. 3B and 4B), MMC-$D_{cat.}$ showed lower activity than MMC against MMC-resistant cells, although MMC-$D_{cat.}$ with molecular weight

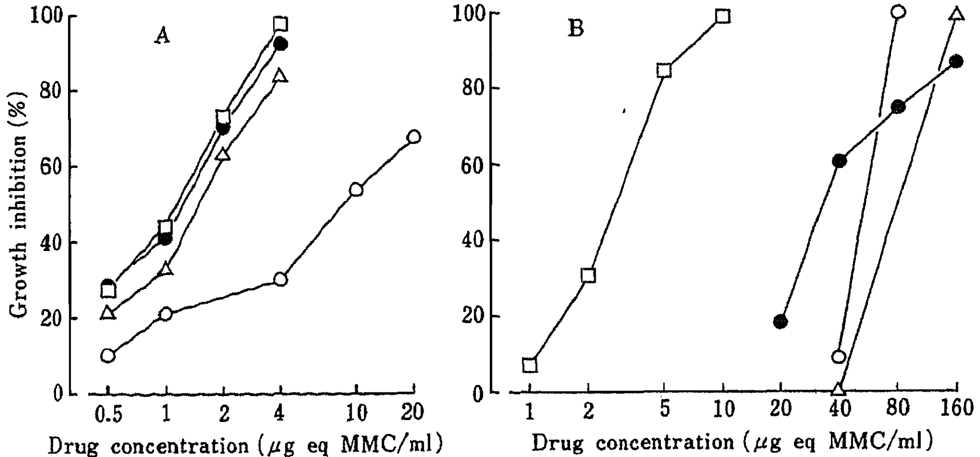

Fig. 4. Growth-Inhibitory Effects of Lipophilic MMC Prodrugs on MMC-Resistant L1210 Leukemia Cell Line (M1) in the Continuous Drug Exposure (A) or 1-h Drug Exposure (B) Experiment

●, MMC; △, benzylcarbonyl MMC; ○, benzyloxycarbonyl MMC; □, nonyloxycarbonyl MMC. Abscissa, logarithmic scale.
A) Growth inhibition was determined as described in the legend to Fig. 3(A).
B) Growth inhibition was determined as described in the legend to Fig. 3(B).

Table II. Sensitivity of MMC-Resistant L1210 Leukemia Cell Lines to Various MMC Prodrugs in the Continuous Exposure Experiment

	IC_{50}[a] (μg eq MMC/ml)			Degree of resistance[b]	
	Parental L1210 (A)	L1210 (M1) (B)	L1210 (M1.5) (C)	(B)/(A)	(C)/(A)
MMC	0.091	1.24	1.38	13.7	15.2
MMC-$D_{cat.}$ (T-10)	0.158	3.24	3.56	21.5	22.5
MMC-$D_{cat.}$ (T-70)	0.153	2.12	3.81	13.9	24.9
MMC-$D_{cat.}$ (T-500)	0.134	2.22	3.54	16.4	26.4
Benzylcarbonyl MMC	0.145	1.48	3.58	10.2	24.7
Benzyloxycarbonyl MMC	0.839	8.71	>20	10.4	>23.8
Nonyloxycarbonyl MMC	0.160	1.15	1.59	7.2	9.9

a) Drug concentration showing 50% growth inhibition. *b*) Calculated as the ratio of IC_{50} of MMC-resistant cells to that of parental cells.

TABLE II. Sensitivity of MMC-Resistant L1210 Leukemia Cell Lines to Various MMC Prodrugs in the 1-h Exposure Experiment

	IC_{50}[a] (μg eq MMC/ml)			Degree of resistance[b]	
	Parental L1210 (A)	L1210 (M1) (B)	L1210 (M1.5) (C)	(B)/(A)	(C)/(A)
MMC	3.6	33.6	60.7	9.3	16.9
MMC-$D_{cat.}$ (T-10)	30.0	123.0	206.9	4.1	6.9
MMC-$D_{cat.}$ (T-70)	0.8	69.3	163.6	86.3	204.5
MMC-$D_{cat.}$ (T-500)	0.4	61.9	156.8	154.8	392.0
Benzylcarbonyl MMC	75.0	81.0	N.D.[c]	1.1	
Benzyloxycarbonyl MMC	35.5	58.0	N.D.	1.6	
Nonyloxycarbonyl MMC	2.1	3.6	5.5	1.7	2.6

a) Drug concentration showing 50% growth inhibition. *b)* Calculated as the ratio of IC_{50} of MMC-resistant cells to that of parental cells. *c)* Not determined.

of 70000 or 500000 showed much higher cytotoxicity against the parental L1210 cells.[16] Among lipophilic prodrugs, nonyloxycarbonyl MMC exhibited higher growth-inhibitory activity than MMC against the MMC-resistant cell line as well as the parental cell line. A close relation between growth-inhibitory activity and cellular uptake of drug was also observed in the case of the resistant cell line.

In Tables II and III, the cytotoxic activities of MMC prodrugs against MMC-resistant L1210 leukemia cell lines are summarized in terms of IC_{50}. In the continuous exposure experiments, MMC-resistant cell lines showed relatively similar resistances to MMC prodrugs and to MMC (Table II). The degree of resistance to nonyloxycarbonyl MMC was somewhat lower than those to other agents.

M1 and M1.5 showed higher resistance to MMC-$D_{cat.}$ as compared with MMC in the 1 h exposure experiments. On the other hand, the degrees of resistance to lipophilic MMC prodrugs, especially nonyloxycarbonyl MMC, were much lower than that to MMC. The IC_{50} value of nonyloxycarbonyl MMC in MMC resistant cell lines remained at the same level as that of MMC with the parental cell line.

Discussion

In cancer chemotherapy, acquisition of resistance to antitumor agents by cancer cells is one of the most serious problems. It was reported that usual response rates to anticancer drugs were only about 35% and the sensitivities of tumor cells to agents which had been previously used for treatment of the patient were significantly decreased in human tumor clonogenic assay.[20,21]

Consequently, many attempts have been made to circumvent drug resistance of tumor cells.[22-24] The resistance of tumor cells to many antineoplastic agents, such as anthracyclines,[25,26] methotrexate,[27] and alkylating agents,[28,29] has been studied. The mechanisms of resistance were considered to involve alteration of the drug transport system in tumor cells (defective cellular accumulation),[30,31] gene amplification of a target enzyme,[32] increase of cellular protective substances such as glutathione,[33] *etc.* Concerning resistance to MMC, the mechanism of resistance in human colon cancer cell lines has been reported to involve a decrease in deoxyribonucleic acid (DNA) cross-link formation,[34] but the details are still unknown.[35]

In this study, we established MMC-resistant cell lines by *in vitro* continuous exposure of

L1210 leukemia cells to MMC and assessed the possibility of overcoming the resistance to MMC by chemical modification of the drug molecule by using this system. The doubling times of resistant cell lines were slightly longer than that of the parental cell line (Table I). The degree of resistance to MMC increased with increase in the concentration of MMC to which the cells had been exposed. The resistance to MMC was stably maintained for more than 6 months. In order to characterize MMC-resistant cell lines, we tested the sensitivity of one (M1) to adriamycin, whose mode of action is rather similar to that of MMC, and resistance to which has been well investigated. The established cell line was also resistant to adriamycin, though MMC-resistant human colon cancer cells were not.[35] This result suggests that M1 has higher activity to repair damaged DNA, and this is responsible for the resistance. In fact, uptake of MMC by resistant cells was not different from that by parental cells.

The mode of action of each type of prodrug tested in this study was previously examined.[16,17] In this investigation, similar results were obtained for the MMC-resistant cell line. MMC-D with its positive charge was strongly adsorbed on the tumor cell surface by electrostatic interaction and an increase in the adsorbed amount of MMC-$D_{cat.}$ with increase of molecular weight was obserbed (Fig. 1). Adsorption of MMC-$D_{cat.}$ on tumor cells conforms to Langmuir's adsorption isotherm.[16] After adsorption on a tumor cell surface, MMC-$D_{cat.}$ releases intact MMC by chemical hydrolysis and the liberated MMC would exhibit cytotoxicity. On the other hand, lipophilic prodrugs were incorporated into tumor cells through partition equilibrium between the medium and cellular lipid components (Fig. 2). After incorporation, lipophilic prodrugs are converted to the parent drug, MMC, by enzymatic hydrolysis.[17] Both types of prodrugs showed a correlation between cytotoxic activity and cellular interaction.

In continuous drug exposure experiments, prodrugs were less active than MMC against the resistant cell lines, and the degrees of resistance to prodrugs were almost the same as that to MMC (Table II). In the 1 h exposure experiment, although MMC-$D_{cat.}$ exhibited higher activity than MMC against the parental L1210 cells, it showed lower activity against MMC resistant cell lines, resulting in higher degrees of resistance (Table III).

In order to inhibit the growth of MMC-resistant sublines, therefore, a higher concentration of MMC was required compared with that in the case of the parental L1210 cell line. The difference in association amounts between MMC-$D_{cat.}$ and MMC was large at low drug concentrations, but it became smaller at a higher concentration where the association of MMC-$D_{cat.}$ was saturated.[16] Thus, prodrugs which show saturable uptake may not be desirable, if large amounts of MMC are required to kill the tumor cells. On the other hand, nonyloxycarbonyl MMC, a lipophilic MMC prodrug, showed higher cytotoxic activity to MMC-resistant cells as well as to parental cells, indicating that chemical transformation could lead to circumvention of drug resistance in this case. From these results, it is suggested that the mode of cellular interaction is important in the manifestation of antitumor effects of prodrugs against drug-resistant cell lines. The reason why the degree of resistance to lipophilic MMC was smaller is not clear.

In vivo, MMC injected in the free form is rapidly cleared from the injection site and the body, and contact with tumor cells occurs for only a short time. The *in vitro* 1-h exposure experiment may refrect this. In this case, MMC prodrugs, especially nonyloxycarbonyl MMC, should interact rapidly and strongly with tumor cells, be retained sufficiently in tumor cells after repeated washing, and show higher growth inhibition even against MMC-resistant tumor cells.

In addition, since the limiting factor in the efficacy of MMC-$D_{cat.}$ against the MMC-resistant cells is saturation of cellular adsorption, MMC-$D_{cat.}$ may be available to overcome resistance in cell lines which are more sensitive to MMC than L1210 leukemia. That is, if an MMC-resistant cell line would be damaged at an MMC concentration which is, of course,

higher than that for the parental cell line but sufficiently lower than that corresponding to saturation of cellular adsorption of MMC-$D_{cat.}$, MMC-$D_{cat.}$ should effectively kill them in the same manner as the parental L1210 cells.[16] In general, L1210 leukemia is known to show lower sensitivity to MMC as compared with other cell lines.[1]

The feasibility of circumvention of the drug resistance by chemical modification of drug molecules to prodrugs having higher affinity to tumor cells has been thus demonstrated.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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Spherical Solid Dispersion Containing Amorphous Tolbutamide Embedded in Enteric Coating Polymers or Colloidal Silica Prepared by Spray-Drying Technique

HIROFUMI TAKEUCHI, TETSUROU HANDA and YOSHIAKI KAWASHIMA*

Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan

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Spherical solid dispersion particles of tolbutamide were prepared by spray-drying a diluted ammonia solution of the drug with additives. Enteric coating polymers (Eudragit® L100-55, hydroxypropyl methylcellulose phthalate) and colloidal silica (Aerosil®) were useful as additives for the solid dispersions. Most of the tolbutamide was dispersed in the amorphous state in the solid dispersion particles when the drug and the additive were formulated in the ratio of 1 : 1. It was a characteristic of the spray-dried solid dispersions that the additive content was smaller than that of dispersions prepared by conventional solvent evaporation or by a grinding method. The drug dissolution *in vitro* was investigated using the disintegration solution No. 2 (pH 6.8) specified in JP X. The dissolution rate from the solid dispersions was markedly improved compared with that from the original or spray-dried drug powder. The rapid dissolution rate from the solid dispersion with enteric coating polymers was attributed to improved wettability and rapid dissolution of the polymer, while that from the dispersion with colloidal silica was found to have a close relationship with the drug crystallinity as well as the wettability.

Keywords——spray-drying; solid dispersion; enteric coating polymer; colloidal silica; tolbutamide; amorphism; wettability; dissolution rate

The use of solid dispersion can improve the solubility or dissolution rate of poorly water-soluble drugs. Various methods such as grinding, coprecipitation, lyophilization and spray-drying are available to prepare solid dispersions, in which the drugs are present in the amorphous state. The spray-drying technique has the desirable characteristics that the resultant particles are spherical and free-flowing. The preparation of spherical particles containing an amorphous drug by using this technique has been investigated by several workers.[1] Recently, Corrigan *et al.*[2] have demonstrated a successful co-spray-drying of a number of thiazide diuretics and non-steroidal antiinflamatory agents with a highly water-soluble polymer, polyvinylpyrrolidone. Fukuda *et al.*[3] have reported that cyclodextrin was a useful carrier for spray-dried solid dispersions.

In this work, spray-dried solid dispersions were prepared by using enteric coating polymers (Eudragit® L100-55, hydroxypropyl methylcellulose phthalate) and a very fine hydrophilic silica particle (Aerosil®) as additives in the formulation. These solid dispersions were found to have good drug-dissolution properties and the amorphous state of the drug was well maintained under dry conditions. The relations between the drug dissolution and the physicochemical properties such as crystallinity of the drug and the wettability of the spray-dried particles to the dissolution medium were investigated. It was found that not only the content of the additives formulated but also the drying rate were closely related to the proportion of the amorphous drug contained in the resultant particles.

Experimental

Material——Eudragit® L100-55 and hydroxypropyl methylcellulose phthalate (HPMCP, HP-55) were ob-

formula 1		formula 2	
tolbutamide	15.0 g	tolbutamide	10.0 g
Eudragit® L100-55	15.0 g	Aerosil® 200	10.0 g
PEG 6000	1.5 g	2% ammonia water	500 ml
Aerosil® 200	3.0 g		
2% ammonia water	500 ml		

Chart 1. Formulae for the Spray-Dried Solution

tained from Röhm Pharma and Shin-Etsu Chemical, respectively. Each enteric coating material has nearly the same dissolution properties depending on the pH of the dissolution solution. Colloidal silica, Aerosil® 200 was a gift from Nippon Aerosil Co., Ltd. Tolbutamide was a gift from Hoechst Japan.

Spray-Drying Technique——The spray-dryer used was a laboratory type one, having a drying chamber 1.2 m in diameter, equipped with a centrifugal wheel atomizer (Okawara, L12 type). Tolbutamide was dissolved in 2% ammonia water and then an enteric coating material or colloidal silica was added to the solution as desired. Typical formulas are listed in Chart 1. In formula 1, a small amount of colloidal silica (Aerosil® 200) was added to improve the micromeritic properties of the resultant particles and polyethylene glycol (PEG) 6000 was added as a plasticizer for the polymer. Unless otherwise stated, the drug- to-additive ratio was 1 : 1. The solution was fed to the spray-dryer by using a roller pump. The spray-drying conditions were as follows: the temperatures at the inlet and outlet of the drying chamber were 140 ± 5 and 95 ± 5 °C, respectively; flow rate of the solution, 1000 ml/h; rotation speed of the atomizer, 16500 rpm. The amount of drying air supplied was controlled to maintain these drying conditions.

Solvent Evaporation Method——The same solution as that for spray-drying was evaporated under reduced pressure by using a rotary evaporator (Yamato, RE46) with a water bath thermally controlled at 45 ± 2 °C. It took more than an hour for the solution, which formed a very viscous liquid, to be completely evaporated. A comparable evaporated sample was obtained by the same procedure but using CH_2Cl_2–EtOH (1 : 1) mixture as a solvent instead of ammonia water. The evaporation was completed within 10 min.

Physicochemical Properties——The shape and surface topography of the spray-dried product were evaluated from scanning electron micrographs (Nihon Denshi, JSM T-20).

The crystallinity of tolbutamide in the spray-dried particles was investigated by X-ray diffractometry (Nihon Denshi, JDX). The crystallinity was calculated by comparing some representative peak heights in the diffraction pattern of the spray-dried particles with those of a physical mixture of the drug and additive formulated at the same ratio. To correct the difference in sample weight in the cell, an internal standard (Al_2O_3) was added to each sample.

Wettability was estimated from the contact angles measured with a contact angle meter (Kyowa Kagaku, CA-A) using compacts of the tolbutamide or spray-dried powder and the dissolution medium saturated with the drug. The compacts were prepared by compressing the powder at 100 kg/cm^2 for 2 min.

Storage Conditions for Stability Test——The samples were kept in a desiccator with silica gel at 30 °C for the dry storage part of the stability test. For the moist storage conditions, the bottom of the desiccator was filled with a saturated aqueous solution of NaCl to maintain 75% relative humidity at 30 °C.

Dissolution Studies——Drug dissolutions of the powdered tolbutamide or the spray-dried products in the disintegration medium No. 2 (pH 6.8, 900 ml) specified in JP X were measured at 37 °C with stirring at 100 rpm by a paddle method. In each dissolution test 300 mg of the sample was used; 3 ml of the dissolution solution was withdrawn at appropriate intervals, and the concentration of tolbutamide in the solution was measured spectrophotometrically at 226 nm by high performance liquid chromatography (HPLC) (Japan Spectroscopic Co., Ltd., Twincle) for the sample including Eudragit or HPMCP, and by spectrophotometry (Hitachi, 100-60) for other samples.

Results and Discussion

Solid Dispersion with Enteric Coating Polymers

Physicochemical Properties——A solid dispersion with enteric coating polymers is called an enteric solid dispersion, and was first described by Hasegawa *et al.*[4] They prepared solid dispersions of several drugs with various kinds of enteric coating polymers by the solvent evaporation method, and showed that in most cases the drugs were present in the amorphous state in the solid dispersions at the drug to polymer ratio of 1 : 3.[5]

In the present study, enteric solid dispersions of tolbutamide were prepared by spray-drying an ammonia solution of the drug and the polymer (1 : 1). As shown in SEM photos (Fig. 1), spherical particles of tolbutamide embedded in the enteric coating polymers were

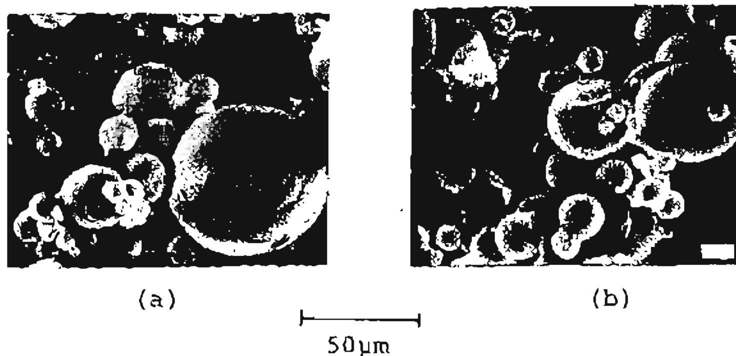


Fig. 1. Scanning Electron Micrograph of Spray-Dried Tolbutamide with Enteric Coating Polymers ((a) Eudragit L100-55, (b) HP-55)

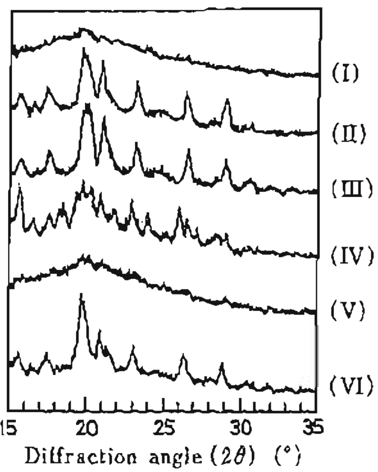


Fig. 2. X-Ray Diffraction Patterns of Spray-Dried Tolbutamide with HP-55 (SD Product) and Related Samples

(I) SD product, (II) physical mixture, (III) evaporated product from an ammonia solution, (IV) evaporated product from an organic solution, (V) SD product stored under dry conditions, (VI) SD product stored under moist conditions. The drug: HP-55 ratio was 1 : 1 in each sample. See the experimental section for the storage conditions. In each case, nearly the same diffraction pattern was observed when Eudragit L100-55 was employed instead of HP-55.

obtained. Infrared analysis indicated that the drug and polymers, which were dissolved in the spray-dried solution, forming the ammonium salts, reverted to their original forms during the spray-drying process. Most of the tolbutamide in these particles was proved to be in the amorphous state on the basis of X-ray diffractometry. While the diffraction patterns of the physical mixture of the drug and the enteric coating polymer (1 : 1) showed the peaks attributed to the drug, only small peaks were observed in the diffraction patterns of the spray dried particles formulated with the drug and polymer at the same ratio (Fig. 2).

It has been shown that the drug-to-polymer ratio is an important factor for the drug transformation to the amorphous state in preparing solid dispersions.[6] The drug-to-polymer ratio in these spray-dried solid dispersions (1 : 1) was larger than that of the solvent-evaporated enteric solid dispersions reported by Hasegawa *et al.*[5] Actually, in preparing the enteric solid dispersion of tolbutamide by the solvent evaporation method, the drug was found to be crystallized without amorphism on the basis of X-ray diffractometry (Fig. 2). This result suggested that the drying rate as well as the drug-to-polymer ratio is an important factor for formation of the amorphous state in the system. Tolbutamide, which was spray-dried from the ammonia solution without polymer, was found to be crystallized without amorphism. Therefore, the use of polymer was essential for the drug to be transformed into the amorphous state in the system, although it was reported that some spray-dried drugs were transformed into the amorphous state without addition of any excipient to the formulation.[7] In conclusion, it was found that the polymer content in the enteric solid dispersion could be reduced by using the spray-drying technique owing to the rapid drying. In

addition to the inherent characteristics of the spray-drying technique, a further advantage of the present method is that those solid dispersions can be prepared without using organic solvents.

Stability Studies——Sugimoto *et al.* have investigated the stability of the amorphous state of drugs in solid dispersions in detail, indicating that this state is sensitive to moisture[8a] and dependent on the type of polymer formulated in the dispersions.[8b] The stability of the amorphous state of the drug in these spray-dried solid dispersions was tested by monitoring the X-ray diffraction patterns of dispersions stored under various conditions as described in the experimental section (Fig. 2). It was found that the amorphous state was well maintained under dry conditions for a month. On the other hand, the diffraction pattern attributable to the drug was observed in the sample stored under moist conditions for the same period. These results suggested that the stability of the amorphous state of the drug in the solid dispersion particle was sensitive to the content of water around or in the sample, although amorphous nifedipine in an enteric solid dispersion was reported to be stable under moist conditions.[9]

Dissolution Studies——Figure 3 shows the dissolution patterns of powdered tolbutamides having various crystal sizes, and spray-dried tolbutamide without the polymer. The dissolution rate of fine crystals of tolbutamide was found to be slower than that of coarse crystals. This was attributed to the formation of agglomerates of fine crystals floating on the surface of dissolution medium, which caused a decrease in the contact area and retarded the dissolution. The dissolution rate of spray-dried tolbutamide was found to be slightly increased, whereas the contact angle of spray-dried tolbutamide (82°) was nearly the same as that of the original powder. The slight increase in the dissolution rate of spray-dried tolbutamide may be due to decreases in both the diameter and amount of agglomerated crystals floating on the surface of the dissolution medium owing to the spherical shape of the spray-dried particles.

As shown in Fig. 4, the dissolution rate of tolbutamide from the solid dispersion particles was very rapid. The rapid drug dissolution from the solid dispersions was partly attributed to their good wettability to the dissolution medium. In measuring the contact angle of the particle, a drop of dissolution medium penetrated into the tablet prepared from the particles in 10—15 s. Thus, the contact angle of the dispersions was not exactly determined, but they were assumed to have good wettability to the dissolution medium in view of the contact angle of tolbutamide (77°). In fact, the solid dispersion particles were well dispersed in the dissolution medium.

In considering the dissolution properties of solid dispersions, an amorphous state of the

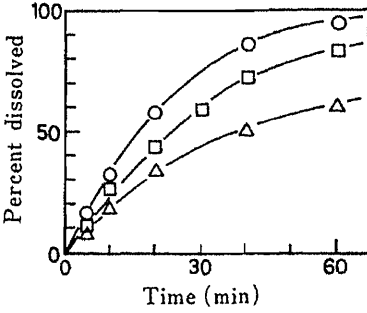


Fig. 3. Dissolution Patterns of Powdered Tolbutamide Having Different Crystal Sizes and Spray-Dried Tolbutamide without Polymer

Powdered tolbutamide (○, original powder; △, −115 mesh); □, spray-dried tolbutamide.

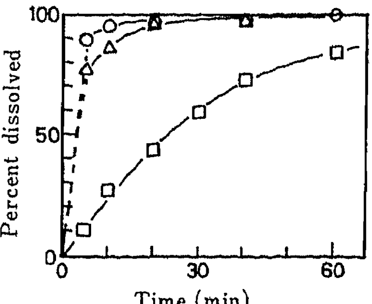


Fig. 4. Drug Dissolution Patterns from the Spray-Dried Solid Dispersion with Enteric Coating Polymers

Solid dispersions (○, with Eudragit L100-55; △, with HP-55); □, original tolbutamide powder.

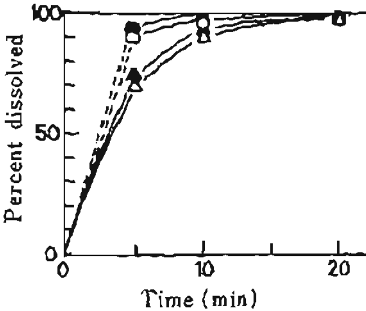
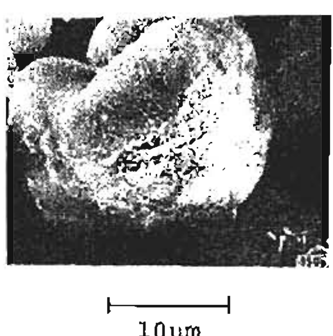


Fig. 5. Effect of the Drug Crystallinity of Solid Dispersions on the Drug Release Patterns

Solid dispersions with Eudragit L100-55 (○, ●) and with HP-55 (△, ▲). Solid dispersions having amorphous drug (open symbols) and crystalline drug (closed symbols) were used.



10 μm

Fig. 6. Scanning Electron Micrograph of Spray-Dried Tolbutamide with Aerosil

The drug: Aerosil ratio was 1 : 1.

drug is an important factor enhancing the drug dissolution rate. The effect of crystallinity on the drug dissolution rate was tested by using two different solid dispersion samples having different drug crystallinities. One sample was the spray-dried particles loaded with amorphous drug and the other, which contained crystalline drug, was prepared by storing the spray-dried sample under moist conditions. As shown in Fig. 5, there was no difference in the drug-dissolution patterns between the two samples. The drug molecules dispersed in the polymer can be immediately dispersed, that is, dissolved in the dissolution medium as the polymer is dissolved. The result shown in Fig. 5 suggested that the crystalline drug in the solid dispersion was dissolved as rapidly as the amorphous drug. This rapid dissolution may occur because the crystalline drug particles are considerably small and can be wetted easily on dissolution of the polymer surrounding them. Consequently, the crystallinity of the drug in these solid dispersions has no effect on the drug dissolution rate, which is presumably limited by the dissolution rate of the polymer contained in the solid dispersion.

In conclusion, the very rapid drug dissolution rate from the solid dispersion was mainly attributed to good wettability, avoiding the effect of agglomeration of particles floating on the surface of the dissolution medium, and to the rapid dissolution of the constituent enteric coating polymer.

Solid Dispersion with Colloidal Silica

Physicochemical Properties——Using an insoluble carrier, a colloidal silica, instead of the enteric coating polymers, spherical solid dispersions were prepared by the spray-drying method. Figure 6 shows a SEM photo of a spray-dried particle. The diameter of the particle was found to be much smaller than that of the original tolbutamide powder and there seemed to be no drug crystals among the spray-dried particles. It was found that no decomposition of the drug occurred during the spray-drying process, based on infrared (IR) and HPLC analyses. The X-ray diffraction patterns shown in Fig. 7 suggested that a large part of the drug was transformed into the amorphous state.

There have been few reports claiming that solid dispersions with such insoluble carriers can be prepared by solvent evaporation methods, including spray-drying method. McGinity and Harris[10] reported a successful preparation of a drug adsorbate having markedly improved drug dissolution properties by using montmorillonite as a carrier with the solvent evaporation method, but they did not refer to the crystallinity of drug adsorbed onto the carrier. Nakai *et al.*[11] reported that an amorphous state was obtained by grinding a drug with an insoluble carrier such as microcrystalline cellulose, cyclodextrin, or some porous materials. They have also investigated the state of the drug dispersed in the ground mixture with

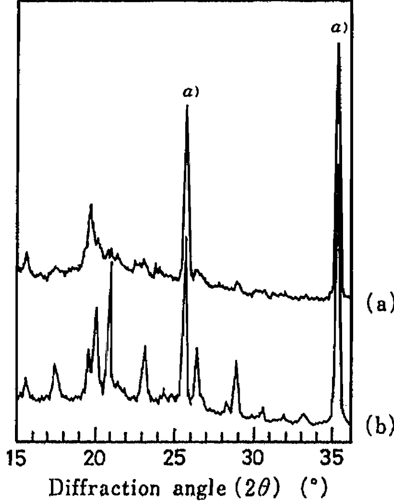


Fig. 7. X-Ray Diffraction Patterns of Spray-Dried Tolbutamide with Aerosil (a) and Physical Mixture of the Drug and Aerosil (b)

The drug: Aerosil ratio was 1 : 1 in each case. The marked peaks *a*) are attributable to an internal standard (Al_2O_3).

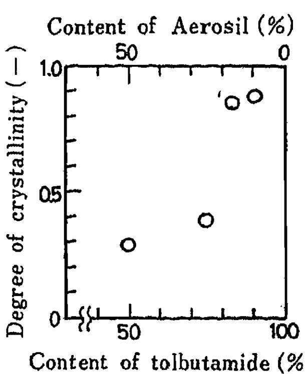


Fig. 8. Change in Crystallinity of Spray-Dried Tolbutamide with Aerosil

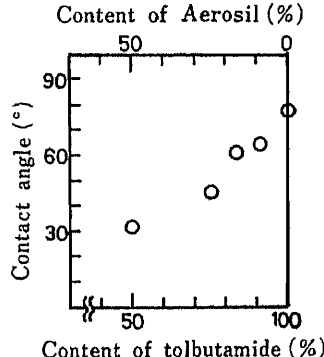


Fig. 9. Change in Contact Angle of Spray-Dried Tolbutamide with Aerosil

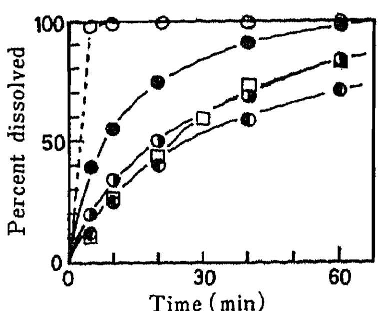


Fig. 10. Drug Dissolution Patterns from the Spray-Dried Particles with Aerosil

The drug: Aerosil ratio was: ○, 1 : 1; ●, 3 : 1; ◑, 5 : 1; ◐, 10 : 1. □, Original tolbutamide powder.

microcrystalline cellulose in detail, suggesting that the drug molecules were present monomolecularly, presumably interacting with cellulose molecules by hydrogen bonding.[11b] In the present solid dispersion, hydrogen bonding between silanol groups of Aerosil and the drug was presumed to play an important role in permitting the drug to be dispersed in the amorphous state.

In the case of the spray-drying method as compared with grinding, it was characteristic that the carrier content in the solid dispersion, in which the drug was present in the amorphous state, was much lower than that in the ground mixture. When the carrier content in the formulation was further reduced, the amount of amorphous drug in the resultant spray-dried particles was decreased. The increasing crystallinity of the drug is shown as a function of the carrier content in Fig. 8. The contact angle of the spray-dried particles was also found to increase with decreasing carrier content, indicating a change in the wettability of the particles (Fig. 9).

Dissolution Studies——The drug dissolution from the solid dispersions was investigated in relation to the physicochemical properties described above. Figure 10 shows the drug dissolution patterns from solid dispersion particles. The solid dispersion containing the carrier and drug in the ratio of 1 : 1, which had good wettability and was loaded with amorphous drug, showed very rapid drug dissolution. The dissolution rate decreased with decrease in the carrier content in the formulation, and the dissolution pattern of the spray-dried product corresponded to that of the original powder when the drug-to-carrier ratio was more than 3. The change in the dissolution patterns described above corresponded to that in both the drug crystallinity and wettability of the solid dispersion shown in Figs. 8 and 9. It is well known that an amorphous drug in a solid dispersion can be dissolved more rapidly than a crystalline drug because of its higher potential energy. The good wettability led to rapid dispersion of the solid dispersion particles in the dissolution medium. Therefore, it was considered that the relationships between the dissolution rate and the physicochemical properties were reasonable.

Conclusion

It was proved that the spray-drying technique is a useful method to prepare solid dispersions with enteric coating polymers or colloidal silica, yielding good drug dissolution properties. By using this technique, spherical particles containing amorphous drug can be directly prepared. It is also a characteristic of this technique that the additive content in the solid dispersion can be reduced compared with that in the case of the conventional solvent evaporation method or grinding. It was found that the drug dissolution properties of the solid dispersions were closely related to their physicochemical properties, such as the drug crystallinity and the wettability.

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Rat Percutaneous Transport of Diclofenac and Influence of Hydrogenated Soya Phospholipids

TOSHIAKI NISHIHATA,*,[1] KEIKO KOTERA, YOHKO NAKANO, and MASARU YAMAZAKI

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan

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Poor penetration of diclofenac through *in vitro* rat dorsal skin including subcutaneous tissue was observed. The poor penetration of diclofenac seemed to be predominantly due to the poor permeability of the stratum corneum. Hydrogenated soya phospholipids (phospholipid) in aqueous gel form increased the penetration of diclofenac in the *in vitro* study, by increasing diclofenac transport through the stratum corneum. In the *in vivo* percutaneous absorption of diclofenac, the presence of phospholipid in aqueous gel form increased both plasma diclofenac concentration and diclofenac accumulation in the dorsal skin tissue, including subcutaneous tissue. Since a marked accumulation of diclofenac in the subcutaneous tissue after application of the aqueous gel was observed both *in vivo* and *in vitro*, percutaneous application of diclofenac in the aqueous gel form, prepared with phospholipid, may be available for topical treatment rather than for systemic treatment.

Keywords——sodium diclofenac; percutaneous transport; *in vitro*, *in vivo* study; rat dorsal skin; subcutaneous tissue; hydrogenated soya phospholipid; aqueous gel; plasma concentration

As described by Osterenga *et al.*,[2] the efficacy of topically applied drugs is often dependent on the composition of the vehicle. The ability of a drug in a topical formulation to penetrate the skin and exert its effect is dependent on two consecutive events. The drug must first diffuse out of the vehicle to the skin surface, and then it must penetrate this natural barrier en route to the site of action. These two processes are intimately related, and both are dependent upon the physical properties of the drug, vehicle, and barrier.

Sodium diclofenac is an effective nonsteroidal antiinflammatory drug. There are few reports on the penetration of sodium diclofenac through the skin, though it has been reported[3] that sodium diclofenac was absorbed well from the rectum.

In the present study, we investigated the penetration of diclofenac through rat skin and the accumulation of diclofenac in subcutaneous tissue in both *in vitro* and *in vivo* experiments. We also examined the effect of hydrogenated soya phospholipids on the penetration of diclofenac. Phospholipids are surfactants, and it has been reported that several surfactants increase the skin permeability.[4] Thus, it is of interest to study the effect of phospholipid as a vehicle component on the penetration of sodium diclofenac.

Experimentals

Materials——Sodium diclofenac was supplied by Ciba Geigy Japan (Takarazuka, Japan). Hydrogenated soya phospholipids (phospholipid), which was suppiled by Nikko Chemicals Co., Ltd. (Tokyo, Japan), contains about 30% phosphatidylcholine and 70% phosphatidylethanolamine, and its iodine value was about 6%. Other reagents used were of analytical grade.

Animals——Male Wistar rats, 200 to 250 g, were fasted for 16 h prior to experiments, but water was given freely. The dorsal hair of rats was shaved with electric clippers. For the *in vitro* study, the dorsal skin was excised just

before an experiment, but the excised skin included subcutaneous tissue.

***In Vitro* Penetration Study**——The *in vitro* penetration study was performed in an LG-1084-LPC penetration cell (Laboratory Glass Apparatus Inc., Berkely, CA, U.S.A.). Briefly, the fluid volume of the receptor side (subcutaneous tissue side) was 5.5 ml, and that of the donor side (stratum corneum side) was 4 ml in the present study. As a fluid, 0.1 M sodium phosphate buffer (pH 7.4) was used. When phospholipid was added to the test solution on the donor side, the solution after addition of phospholipid was homogenized at 80 °C for 10 min to accelerate the hydration of phospholipid to produce the aqueous gel form.[5] The skin membrane surface area exposed to fluid was 530 mm^2 (26 mm diameter). Fluid on the receptor side was kept at 37 °C.

After settlement of the skin into the apparatus, 4 ml of test solution containing sodium diclofenac at a concentration of 1.25 mg/ml was placed on the donor side, and then 100 μl samples were collected from the receptor side at 1 h intervals for 7 h. Just after collection of fluid, 100 μl of buffer solution was added to keep the volume constant, fluid on the receptor side was agitated with a magnetic stirrer.

In another series of experiments, 5.5 ml of the test solution or the aqueous gel (0.25%(w/w)phospholipid) containing sodium diclofenac was placed on the receptor side (subcutaneous tissue side) to investigate the distribution of diclofenac from the buffer solution to the subcutaneous tissue. After the solution of diclofenac was placed on the subcutaneous side, 20 μl samples were collected from the receptor side at 5, 15, 30, 45, 60 min, and 2 h.

To measure accumulation of diclofenac in the tissue, the tissue was homogenized, following the rinsing of the skin tissue with saline after an *in vitro* penetration study. After addition of acetonitrile to the homogenate and centrifugation, the supernatant was collected and then dried under a flow of nitrogen gas. The residue was dissolved in a mixed solvent of 25%(v/v) acetonitrile and 75%(v/v) 0.05 M citrate buffer (pH 5.5), which was used as a mobile phase in high-performance liquid chromatography(HPLC).

***In Vivo* Absorption Study**——A rat was anesthetized with sodium pentobarbital (30 mg/kg, i.p., for the first injection, and then 15 mg/kg at 2.5 h intervals during the experimental period), and placed on a hot surface at 38 °C. The administration of the test solution was performed with a cylinder (26 mm inner diameter and 20 mm high), which was fixed perpendiculary on the rat dorsal skin surface with glue (Aron Alpha®, obtained from Toa Chemicals Co., Ltd., Tokyo, Japan). After the administration, 200 μl of blood was collected from the right femoral vein *via* a canuula at designated time intervals for 12 h. After 12 h, the skin tissue including subcutaneous tissue on which the test solution had been applied was excised to measure the accumulation of diclofenac in the tissue. The intravenous administration of diclofenac solution was performed *via* the jugular vein.

Assay——Assay of sodium diclofenac was carried out by HPLC as described by Yaginuma *et al.*[6] The lowest concentration of diclofenac that could be determined was 40 ng/ml.

Statistical Analyses——Statistical analyses were performed by means of Student's *t*-test.

Results and Discussion

In Vitro Penetration of Diclofenac through Rat Skin Including Subcutaneous Tissue

Penetration of diclofenac through the skin was poor when the test solution on the donor side contained sodium diclofenac at a concentration of 1.25 mg/ml (Fig. 1A). After 7 h, only about 0.015% of the diclofenac was recovered on the receptor side. The application of the aqueous gel, prepared with phospholipid at various contents, increased the penetration of diclofenac, in proportion to phospholipid content (Fig. 1A). However, contents of phospholipid above 0.5% (w/v) did not further increase diclofenac penetration.

Since the accumulation of diclofenac in the subcutaneous tissue is an important factor for topical therapy, accumulation of diclofenac in the tissue was investigated at 2, 5 and 7 h after placing the test solution on the stratum corneum (Fig. 1B). The amount of diclofenac accumulated in the tissue increased with increasing incubation time. The accumulation of diclofenac also increased in the presence of the aqueous gel of phospholipid. The apparent penetration rate of diclofenac at 2, 5 and 7h was determined by appling the following equation:

$$\text{apparent penetrate rate} = [\text{increase of diclofenac on the receptor side from } (t)\text{ h to } (t+\Delta t)\text{ h}]/[(\Delta t)\text{ h}]$$

where t and $t+\Delta t$ are times (h) after starting the experiments. In the persent study, the values of the apparent penetration rate were determined from the increase of diclofenac amount on the receptor side from 1 to 3 h for the rate at 2 h, from 4 to 6 h for the rate at 5 h, and from 6 to

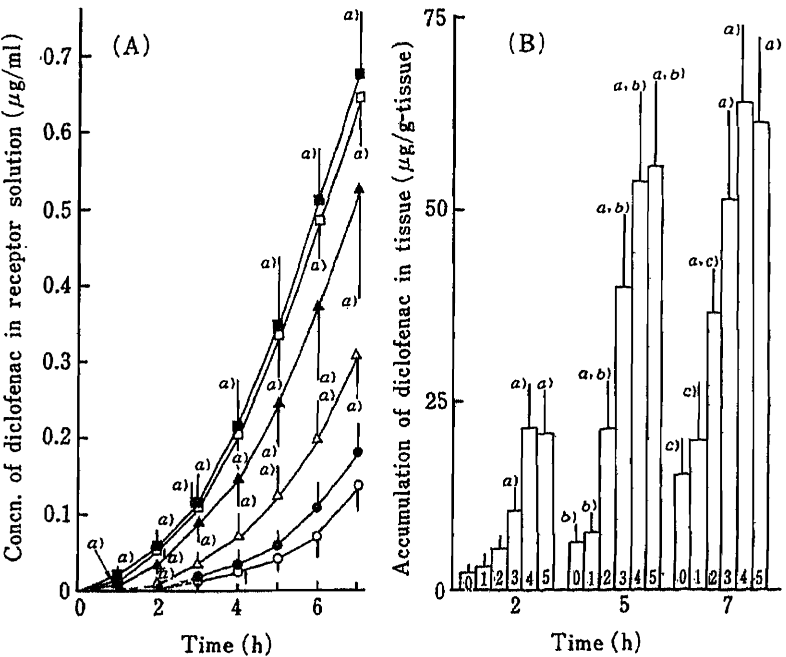

Fig. 1. *In Vitro* Penetration of Diclofenac from the Stratum Corneum Side through Rat Skin Including Subcutaneous Tissue (A) and Accumulation of Diclofenac in the Tissue (B)

The test solution (4 ml) or the aqueous gel, containing 1.25 mg sodium diclofenac/ml, was placed on the stratum corneum: ○ in (A) and 0 in (B), the solution (no phospholipid); ● and 1, the gel with 0.05% (w/v) phospholipid; △ and 2, the gel with 0.1% (w/v) phospholipid; ▲ and 3, the gel with 0.25% (w/v) phospholipid; □ and 4, the gel with 0.5% (w/v) phospholipid; ■ and 5, the gel with 1% (w/v) phospholipid. Wet weight of the skin tissue used was from 1.97 to 2.42 g. Each value represents the mean ± S.D. ($n=3$ to 5). *a*) $p<0.05$ *versus* no phospholipid; *b*) $p<0.05$ *versus* 2 h; *c*) $p<0.05$ *versus* at 5 h.

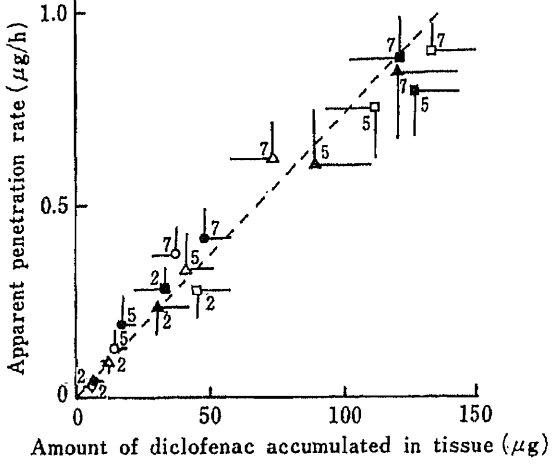

Fig. 2. Relationship between Apparent Penetration Rate of Diclofenac (Determined by the Method Described in the Text) and Total Amount of Diclofenac Accumulated in Subcutaneous Tissue at 2 h (2), 5 h (5) and 7 h (7)

The data in this figure were obtained from Fig. 1. Symbols are as follows: ○, no phospholipid; ●, 0.05% (w/v) phospholipid; △, 0.1% (w/v) phospholipid; ▲, 0.25% (w/v) phospholipid; □, 0.5% (w/v) phospholipid; ■, 1% (w/v) phospholipid. Accumulation of diclofenac in the tissue represents the total amount in the tissue. Each value represents the mean ± S.D. ($n=3$ to 5).

7 h for the rate at 7 h. As shown in Fig. 2, there was a good relationship between the apparent penetration rate and the total amount of diclofenac accumulated in the skin tissue.

These results indicate that an increase of the penetration rate of diclofenac through the skin occurred along with an increase of diclofenac accumulation in the subcutaneous tissue. In another series of experiments, the distribution of diclofenac to the subcutaneous tissue was examined. When test solution containing sodium diclofenac at various concentrations was placed on the subcutaneous tissue side, distribution of diclofenac into the tissue occurred rapidly, as represented by the decrease of diclofenac concentration in Fig. 3A. This result

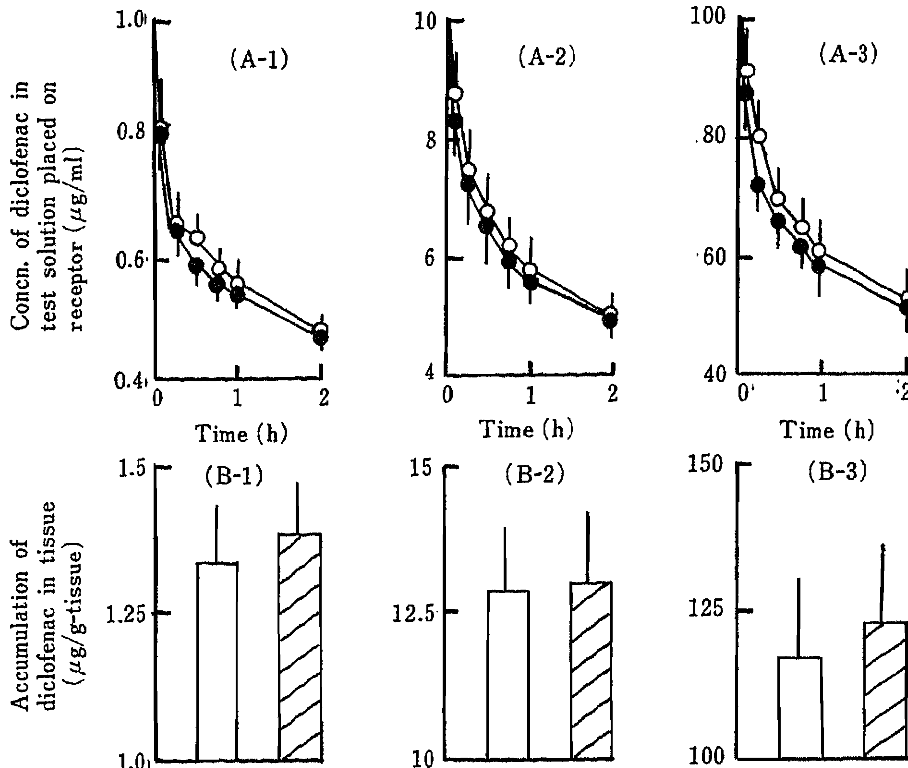

Fig. 3. Distribution of Diclofenac from Test Solution into Subcutaneous Tissue in an *in Vitro* Rat Study, when the Solution or the Aqueous Gel (0.25% (w/v) Phospholipid) was Placed on the Subcutaneous Side

(A) Concentration of diclofenac in the test solution on the subcutaneous side (5.5 ml) as a function of time. Initial concentrations of diclofenac in the test solution in the presence (closed symbols) and in the absence (open symbols) of phospholipid at 0.25% (w/v) were as follows: (A-1), 1 μg/ml; (A-2), 10 μg/ml; (A-3), 100 μg/ml.

(B) The total amount of diclofenac accumulated in the tissue excised at 2h after incubation. Initial diclofenac concentration in the test solution in the presence (▨) or in the absence (□) of 0.25% (w/v) phospholipid were as follows: (B-1), 1 μg/ml; (B-2), 10 μg/ml; (B-3), 100 μg/ml. Wet weight of the tissue excised was 2.21 to 2.54 g. Each value represents the mean ± S.D. ($n=3$).

indicates that slow uptake of diclofenac into the skin tissue from the stratum corneum side is due to the low permeability of stratum corneum to diclofenac. When the aqueous gel, prepared with phospholipid at 0.25% (w/v), was placed on the subcutaneous side, the distribution of diclofenac into the subcutaneous tissue did not change greatly in comparison with that the absence of phospholipid (Fig. 3). The ratio of amount of diclofenac accumulated at 2h to initial diclofenac concentration was not affected by the initial concentration of diclofenac or by the presence of phospholipid.

These observation seem to indicate that the increase of diclofenac accumulation in the tissue from the stratum corneum in the presence of phospholipid (Fig. 1B) occurs through an acceleration of diclofenac penetration through the stratum corneum by phospholipid, since phospholipid did not influence the distribution of diclofenac into the subcutaneous tissue. Although we did not investigate in detail how phospholipid increased the permeability of the stratum corneum to diclofenac, it may be supposed that a surfactant effect of phospholipid is involved, since it has been reported that extraction of lipid from the stratum corneum increased the permeability[7] and several surfactants could extract the lipid.[4] Recently, Natsuki *et al.*[8] have reported that indomethacin gel ointment containing egg lecithin gave enhanced transdermal absorption of indomethacin from rat dorsal skin. Thus, it is considered

that phospholipids in the aqueous gel increase the permeability of the stratum corneum of rat dorsal skin.

In Vivo Percutaneous Absorption of Diclofenac in Rat

In the *in vivo* percutaneous absorption study, two application forms were examined; the solution and the aqueous gel containing phospholipid at 0.5% (w/v). After intravenous administration of diclofenac into rat jugular vein, elimination of diclofenac occurred rapidly, as shown in Fig. 4B.

After the administration of diclofenac solution or diclofenac aqueous gel, diclofenac appeared in the plasma, and the plasma concentration of diclofenac was maintained roughly

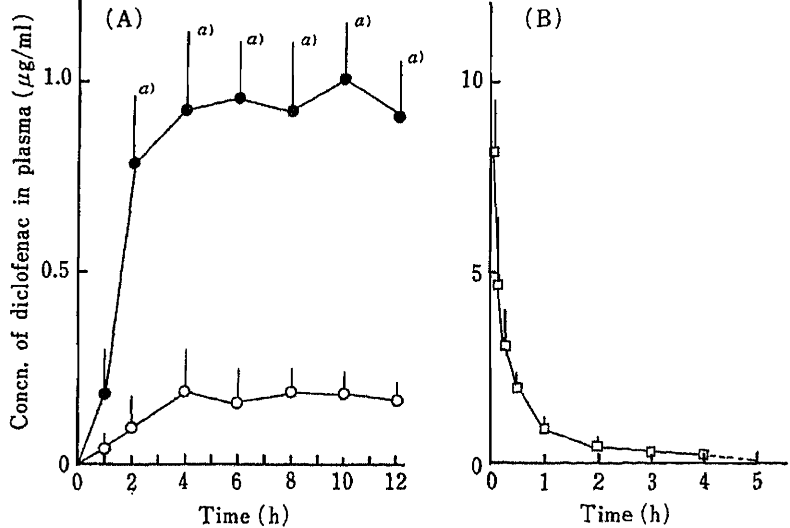

Fig. 4. Plasma Diclofenac Concentration in Rats as a Function of Time after the Percutaneous Administration (A) of 4 ml of the Solution (Open Circles) or 4 ml of the Aqueous Gel (Closed Circles, 0.5% (w/v) Phospholipid) at a Dose of 5 mg of Sodium Diclofenac (1.25 mg of Sodium Diclofenac/ml in Test Sample) and after Intravenous Administration (B) of Diclofenac at a Dose of 0.5 mg/rat

Each value represents the mean ± S.D. ($n=4$). *a*) $p<0.05$ *versus* the solution.

TABLE I. Area under the Curve of Plasma Diclofenac Concentration (*AUC*) for 12 h after Administration and Total Amount (AMOU) of Diclofenac Accumulated[a] in the Dorsal Skin Tissue Including Subcutaneous Tissue, after Intravenous Administration or Percutaneous Administration of Sodium Diclofenac in Rats

	Dose (mg/rat)	*AUC* (μg h/ml)	BA[b] (%)	AMOU[a] (μg)	Wet weight of tissue[c] (g)
Intravenous administration	0.5	4.24±0.47	100	Undetectable	2.52±0.41
Percutaneous administration					
Solution	5.0	1.82±0.56	4.29	11.6±6.2	2.61±0.37
Aqueous gel[d]	5.0	10.86±3.77[e]	25.61	96.2±31.7[e]	2.29±0.24

a) The amount of diclofenac accumulated in the tissue was measured at 0.5 h after intravenous administration, and at 12 h after percutaneous administration. *b*) BA represents bioavailability, which was determined as follows:

$$BA=(AUC)_{skin}(dose)_{i.v.}/(AUC)_{i.v.}(dose)_{skin}$$

where skin and i.v. represent percutaneous administration and intravenous administration, respectively. *c*) Wet weight of the skin tissue excised to measure the accumulation of diclofenac. *d*) Aqueous gel containing 0.25% (w/v) phospholipid. Each value represents the mean ± S.D. ($n=4$). *e*) $p<0.05$ *versus* solution.

from 2 to 12 h during the experimental period (Fig. 4A). The plasma diclofenac concentration obtained by the application of aqueous gel was significantly greater than in the case of the solution. The appearance of diclofenac in plasma seems to be rapid in comparison with the *in vitro* penetration study. Since it is considered that blood flow systems in the rat reach close to epidermis, as in humans,[9] diclofenac after penetration through the stratum corneum passes into the blood flow system after diffusion through the thin epidermis layer. However, the skin including subcutaneous tissue in the *in vitro* study represents a long diffusion layer before reaching the receptor solution; *i.e.*, a long lag time was observed in the *in vitro* study before appearance of diclofenac in the receptor solution.

The amounts of diclofenac accumulated in the skin tissue region including subcutaneous tissue were greater when the aqueous gel was applied than in the case of the solution (Table I). This result is consistent with the *in vitro* study; *i.e.*, marked accumulation of diclofenac in the tissue caused a higher concentration of diclofenac in plasma. Thus, it is considered that the amount of diclofenac accumulated in the tissue under the skin can be related to plasma concentration of diclofenac. In terms of intravenous administration of diclofenac, when the plasma diclofenac concentration was more than 2 μg/ml (Fig. 4B), accumulation of diclofenac in the subcutaneous tissue of the skin was not detectable (Table I).

The bioavailability of diclofenac in percutaneous absorption, which was determined evaluating the area under the curve (*AUC*) of plasma diclofenac concentration for 12 h, in comparison with that after intravenous administration, was 5% for the solution and 25% for phospholipid aqueous gel (Table I). It should be noted that a marked accumulation of diclofenac in the skin tissue was observed (Table I), in spite of the low bioavailability of diclofenac in plasma, after application of the aqueous gel form seems to be available for topical treatment rather than for systemic treatment.

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Production and Physicochemical Properties of Water-Insoluble Glucan from *Streptococcus mutans*

KEN MASUMOTO,*[,a] KUMIKO YAMASHITA,[a] AKIYOSHI YOSHIDA,[a] SHIN'ICHI HAYASHI,[b] YOSHIHARU MACHIDA,[c] and TSUNEJI NAGAI[c]

The Application and Development Division,[a] the Research and Development Division[b] of Rohto Pharmaceutical Co., Ltd., Tatsumi Nishi-1-8-1, Ikuno-ku, Osaka 544, Japan, and Faculty of Pharmaceutical Sciences, Hoshi University,[c] Ebara-2-4-41, Shinagawa-ku, Tokyo 142, Japan

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A method for the production of water-insoluble glucan was developed by utilizing the extracellular glucosyltransferase present in the culture fluid of *Streptococcus mutans*, and some physicochemical properties of the glucan powder were investigated.

Streptococcus mutans was cultured in brain heart infusion (BHI) medium. The growth phase shifted to the logarithmic phase and the stationary phase at 6 and 14 h after the inoculation of the preculture into BHI medium, respectively. The doubling time was about 180 min.

Water-insoluble glucan was produced by incubation of the cell-free supernatant of the culture fluid with sucrose added as a substrate. The amount of water-insoluble glucan produced was affected by the concentration of substrate, temperature and pH; the optimum values of these factors were 15% (w/w), 37 °C and 7.5, respectively. By gas-liquid chromatographic analysis of the methylated water-insoluble glucan, the glucan chain was found to consist of 60% of α-1,3- and 20% of α-1,6-D-glucosidic bonds. The molar percent of each type of linkage was not affected by the pH of the reaction mixture but was affected by the incubation temperature. Among 8 strains examined, OMZ-176 and 6715 showed the best production of water-insoluble glucan. No peak was observed in the powder X-ray diffraction pattern of the freeze-dried glucan, suggesting that it is an amorphous powder. The hygroscopicity of the glucan powder was similar to that of corn starch. A rheological study showed that a dispersion of the glucan had non-Newtonian and shear-thinning behavior.

Keywords——production method; water-insoluble glucan; *Streptococcus mutans*; physicochemical property; extracellular glucosyltransferase

It has been known for more than twenty years that streptococci have cariogenic action. These cariogenic streptococci are generally called *Streptococcus mutans*. These microorganisms produce water-soluble or insoluble polysaccharides such as glucan and fructan from sucrose by the action of extracellular enzymes.[1,2] Recently water-insoluble glucan has been shown to play an important role in the formation of dental plaque, and many studies on the chemical properties[3,4] and crystal structure[5] of glucan have been reported. There have also been many reports concerning the characteristics of glucosyltransferases which produce water-insoluble glucan from sucrose as a substrate.[6-8] However, there are only a few reports concerning the utilization of this glucan.

Starch and cellulose are well known polysaccharides obtained from plants, and are widely used, intact or as their derivatives, in many fields. On the other hand, dextran is a polysaccharide obtained by using microorganisms. Moreover, it has been confirmed that some polysaccharides produced by microorganisms have unique characteristics different from those of the synthetic polymers. Some of those polysaccharides, *e.g.*, pullulan[9] and curdlan,[10]

which have anomalous flow characteristics, have been put to practical use.

As a part of our investigations concerning the pharmaceutical usefulness of water-insoluble glucan, we have reported that glucan is useful as a vehicle for directly compressed tablets[11] and sustained-release tablets.[12] However, there is no report describing a practical production method. Therefore, in this study, the effects of concentration of substrate, pH and strain of *Streptococcus mutans* on the production of water-insoluble glucan were investigated. Moreover, some physicochemical properties of water-insoluble glucan are presented.

Experimental

Bacterial Strains——*Streptococcus mutans* strains OMZ 176, OMZ 175, OMZ 65, 6715, B 13, MT-615R, EN 19-1 and E 49 were used in this study. All strains were obtained from the Department of Microbiology, Dental School, Osaka University, Osaka, Japan.

Cultural Conditions——The organism was cultured under aerobic conditions in batch culture at 37 °C for 18 h using Brain Heart Infusion Broth (Difco Laboratories, Detroit, Mich.). Preculture was carried out in the same medium for about 24 h. Growth was determined by measuring the absorbance of the culture at 570 nm with a spectrophotometer (UV-240, Shimadzu Corp., Kyoto, Japan).

Production and Fractionation of Glucan——Glucan was produced and fractionated by means of the procedures shown in Chart 1. The culture was harvested in the stationary phase, *i.e.*, 18 h after inoculation, and the cell-free supernatant was obtained by centrifugation at 3000 × *g* for 10 min at 4 °C (KR-2000T, Kubota Medical Appliance Supply Corp., Tokyo, Japan). Unless otherwise stated, the supernatant fraction was incubated at 37 °C for 4—7 d with 15% sucrose and 0.04% sodium azide. The water-insoluble glucan was isolated by centrifugation, washing by distilled water several times and freeze-dried. The water-soluble glucan in the supernatant fraction was fractionated by precipitation with a final concentration of 80% ethanol. The ethanol-insoluble fraction was isolated by centrifugation, washed with 80% ethanol and freeze-dried.

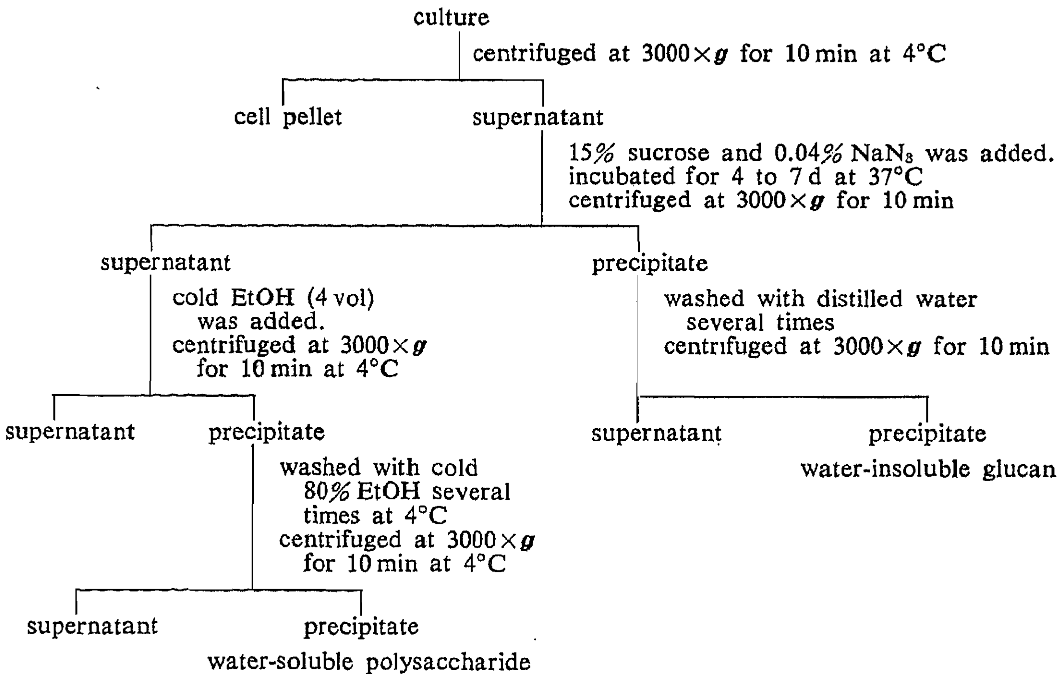


Chart 1. Procedure for Production and Fractionation of Polysaccharides from *S. mutans*

Determination of Glucan and Individual Saccharides in the Reaction Mixture——Water-soluble and insoluble glucan isolated were measured by the phenol sulfuric acid method.[13] Sucrose, glucose and fructose in the reaction mixture were measured by gas-liquid chromatography (GLC) using sorbitol as an internal standard. Cold ethanol (4 volumes) was added to the reaction mixture at 4 °C and the precipitate was removed. The supernatant was diluted with 9 volumes of water and a 1 ml aliquot was evaporated *in vacuo* in a rotary evaporator at 30 °C. The dried sample was treated with 0.5 ml of trimethylsilylating reagent (pyridine: hexamethyldisilazane: trimethylchlorosilane=

10:5:1) for 30 min at room temperature and analyzed by GLC. The GLC apparatus and operating conditions were as follows: instrument, model GC-7A (Shimadzu Corp., Kyoto, Japan); column, 5% silicone GE SE 30, Chromosorb W (AW-DMCS), 3.2 mm × 1.0 m; column temperature, a linear gradient of 170—250 °C at 8 °C/min from 16 min after injection; detector, FID; detector and injection temperatures, 270 °C; carrier gas, N_2, 50 ml/min; H_2 and air pressure, 0.5 kg/cm^2.

Methylation Analysis——Ten milligrams of the glucan dried *in vacuo* over phosphorus pentoxide was suspended in 4 ml of dimethyl sulfoxide at 60 °C in a nitrogen atmosphere, then methylated by the method of Hakomori[14] with 0.5 ml of fresh methylsulfinyl carbanion. The reaction mixture was allowed to stand for 5 h at room temperature, then 1 ml of methyl iodide was added under cooling in an ice bath. The reaction mixture was dialyzed against water overnight at room temperature and the methylated glucan was extracted into chloroform. The extract was evaporated *in vacuo* using a rotary evaporator. The methylated glucan was hydrolyzed with 0.2 ml of 72% sulfuric acid for 1 h at 0 to 5 °C, then for 6 h at 100 °C after dilution with 1.2 ml of water. The hydrolyzate was neutralized with barium carbonate. The methylated sugar components were reduced with 20 mg of sodium borohydride, converted into alditol acetates by treatment with 0.2 ml of pyridine and 0.2 ml of acetic anhydride for 2 h at 100 °C, and dried *in vacuo* using a rotary evaporator. The acetylated glucan was dissolved in chloroform and an aliquot was analyzed by GLC. The apparatus and operating conditions of GLC were as follows: instrument, model GC-7A (Shimadzu Corp., Kyoto, Japan); column, 3% ECNSS-M, Gaschrom Q, 3.2 mm × 2.1 m; column temperature, 190 °C; detector, FID; detector and injection temperatures, 300 °C; carrier gas, N_2, 60 ml/min; H_2 and air pressure, 0.5 kg/cm^2.

Infrared (IR) Spectroscopy——Measurement was carried out by the KBr disc method using an infrared spectrophotometer (model 260-10, Hitachi, Tokyo, Japan).

Powder X-Ray Diffractometry——Powder X-ray diffractometry was carried out using a diffractometer (Geigerflex model D-2, Rigaku Denki, Tokyo, Japan) with Ni-filtered, Cu-K_α radiation.

Study on Flow Characteristics of Glucan Dispersion——Glucan gel (6% (w/w)) was prepared by mixing the glucan and water in a mortar with a pestle, and allowed to stand overnight at 4 °C. Assessment of the flow characteristics of the glucan dispersion was carried out at 20 °C using a rotational viscometer, Haake Rotovisko RV12 (Haake Mess-Technik GmbH) equipped with an M 500 measuring head and MV II sensor system.

Results and Discussion

The production of water-insoluble glucan (WIG) has been observed in many strains of *Streptococcus mutans* (*S. mutans*). In this study we investigated the yield and the nature of WIG produced by several strains of *S. mutans* from the viewpoint of potential pharmaceutical application. The yields of WIG and the water-soluble polysaccharide (WSP) obtained from 8 strains of *S. mutans* are shown in Table I. The yields of polysaccharides varied from strain to strain. Among 8 strains, OMZ 176 and 6715 produced most WIG. In addition, WIG obtained

Table I. Polysaccharide Production by Various Strains of *S. mutans*

Strain	Yield of WIG (g/l)	Yield of WSP (g/l)
OMZ 176	4.0±0.6	13.9±1.1
OMZ 175	0.7±0.2	17.0±2.0
OMZ 65	2.2±0.3	10.0±0.9
6715	4.0±1.0	7.4±1.5
B-13	1.6±0.6	9.7±0.6
MT-615R	0.6±0.5	13.3±2.4
EN 19-1	0.3±0.2	33.8±5.4
E-49	1.5±0.9	8.9±2.6

Production was carried out for 96 h at 37 °C. Each value represents the mean ± S.D. of ten and three determinations for OMZ-176 and other strains, respectively.

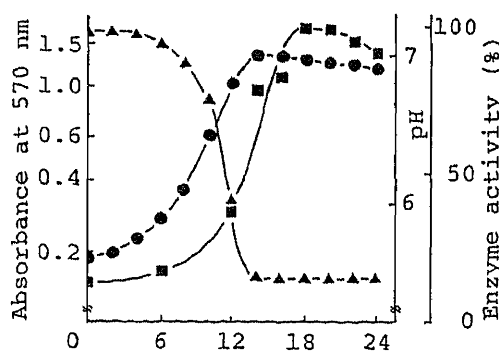


Fig. 1. Changes of Enzyme Activity and pH during Growth

●, absorbance at 570 nm; ■, enzyme activity in the culture fluid; ▲, pH of the culture fluid. The degrees of relative activity are expressed as a percentage of the activity of culture fluid obtained 18 h after inoculation. Each point represents the mean of three determinations.

from OMZ 176 according to the procedure shown in Chart 1 was pulverized most easily, and gave a white powder on freeze-drying. We have previously reported the pharmaceutical availability of WIG from OMZ 176,[11,12] so, OMZ 176 was selected as the preferred strain for all subsequent experiments in this investigation.

Changes of pH and the Enzyme Activity of the Culture Fluid during Growth

Figure 1 illustrates the changes of pH of the culture fluid and the activity of the extracellular glucosyltransferase (GTase) in the culture fluid during growth of the organism. The growth phase shifted to the logarithmic phase and to the stationary phase at 6 and 14 h after the inoculation of the preculture, respectively. The doubling time at the log phase was about 180 min. The initial pH of the culture fluid was 7.2. The pH of the culture fluid decreased with growth and remained at 5.5 after the temperature of growth. On the other hand, the activity of the extracellular GTase in the culture fluid increased with growth after a lag time of about 3 to 6 h. The maximum activity of GTase was observed at 4 h after the termination of growth, *i.e.*, 18 h after the start of the culture. Therefore, in subsequent experiments, the cell-free culture fluid containing GTase was harvested, in principle, 18 h after the start of the culture and utilized as the crude enzyme solution.

Production of the WIG

Time Course of WIG Production——The time course of the production of WIG is shown in Fig. 2. The production of WIG proceeded in proportion to incubation time for about 96 h. However, the rate of production slowed down suddenly at 96 h after the start of the incubation. The WIG preponderantly adhered to the glass surfaces of the vessel in an agglomerated state, and hardly any was observed suspended in the reaction mixture. Dispersion of the adhered WIG by agitation was not easy.

Chemical Changes in the Reaction Mixture during WIG Production——Changes of the concentrations of sucrose, glucose and fructose in the reaction mixture during the production of WIG are shown in Fig. 3. The consumption of the substrate, *i.e.*, sucrose, corresponded quite well to the total production of WIG, WSP, glucose and fructose. A moderate amount of glucose that corresponded to 25% of the sucrose consumed was observed. Gibbons reported

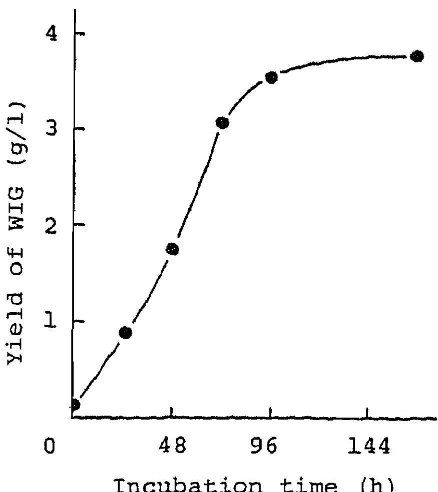


Fig. 2. Time Course of WIG Production

The reaction mixture used was obtained 18 h after the inoculation of the preculture. The pH of the reaction mixture was not regulated. The incubation temperature was 37 °C and the concentration of sucrose was 15%. Each point represents the mean of three determinations.

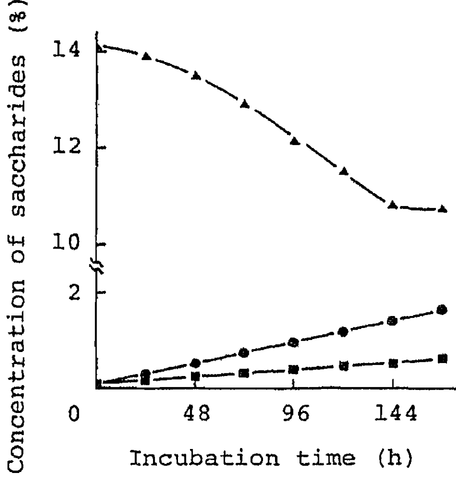


Fig. 3. Chemical Changes in the Reaction Mixture during Polysaccharide Production

▲, sucrose; ■, glucose; ●, fructose. The conditions of production were the same as in Fig. 2. Each point represents the mean of three determinations.

that a cell-associated invertase-like enzyme, which appeared to be similar to invertase on the basis of its catalytic action, its molecular size, and its heat sensitivity, was present in cells of *S. mutans* strains GS 5 and 6715.[15] The same invertase-like enzyme might exist in the culture fluid. On the other hand, glucanase might also be present in the culture fluid. Therefore, the production of glucose might have been due to the action of an invertase-like enzyme and/or glucanase. On the other hand, the production of fructose should be mainly due to the action of the GTase, since the amount of fructose produced was much larger than that of glucose. In addition, it was suggested that the production of water-soluble fructan was small since the amount of fructose produced was about 85% of that calculated from the amount of sucrose consumed.

Effect of Initial Substrate Concentration on WIG Production——The effect of initial substrate concentration on WIG production is shown in Fig. 4. When the cell-free culture fluid or the crude enzyme solution, *e.g.*, a phosphate buffer in which 50% ammonium sulfate-saturated fraction of culture fluid was dissolved,[3] was used as the reaction mixture for WIG or WSP production, the concentration of sucrose applied was reported to be 10%. On the other hand, when the WIG and WSP production was carried out during culture, the concentration of sucrose applied was reported to be 5%.[16] However, we found that the amount of WIG produced was largest when the initial concentration of sucrose was 15%.

Effect of pH on WIG Production——The effects of pH of the reaction mixture on WIG production and on the structure, *i.e.*, molar ratios of linkage types, of WIG produced are shown in Fig. 5 and summarized in Table II, respectively. The amount of WIG produced was the largest when the pH of the reaction mixture was 7.5. The amount of WIG produced at pH 7.5 was about 1.7 times that produced when the pH was not regulated, *i.e.*, pH 5.5. On the other hand, the molar ratios of linkage types of WIG produced at each pH were essentially the same. In addition, the ratios were comparable to those reported for WIG produced by strain OMZ 176.[3,17,18]

GTase has been reported to consist of several components.[6-8] Guggenheim and Newbrun reported that the optimum pH of those components fell in the range of pH 5.0 to

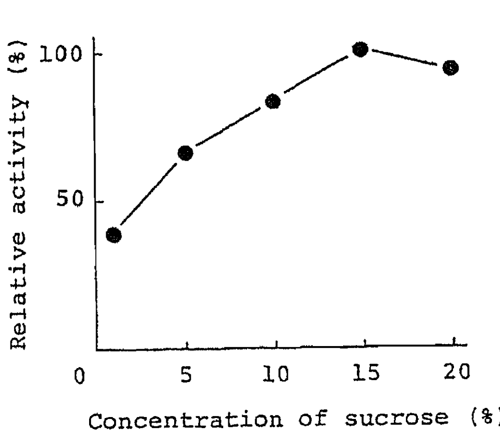


Fig. 4. Effect of Initial Substrate Concentration on WIG Production

The reaction mixtures containing sucrose at various concentrations were incubated at 37 °C for 168 h. The degrees of relative activity are expressed as a percentage of the activity at the concentration of 15% sucrose. Each point represents the mean of three determinations.

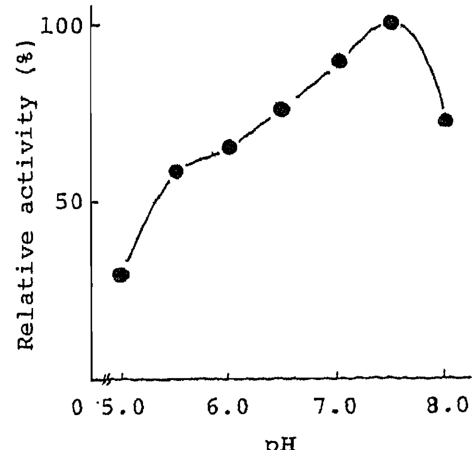


Fig. 5. Effect of pH of the Reaction Mixture on WIG Production

The pH of each reaction mixture was adjusted with McIlvaine's buffer and confirmed not to have changed after WIG production by using a pH meter. Each reaction mixture was incubated for 96 h at 37 °C. The degrees of relative activity are expressed as a percentage of the activity at pH 7.5. Each point represents the mean of three determinations.

TABLE II. Effect of pH of the Reaction Mixture on WIG Structure

pH	Methyl *O*-methyl-D-glucopyranoside			
	2,3,4,6-Tetra-	2,4,6-Tri-	2,3,4-Tri-	2,4-Di-
5	11±0.6	62±0.6	17±0.6	10±0.6
6	10±0.6	63±2.3	16±2.1	11±1.2
7	13±2.5	57±2.1	19±1.2	11±1.5
8	9±1.2	62±1.5	19±2.0	10±1.0

Each value represents the mean molar percent±S.D. of three determinations. Each WIG was produced at 37 °C.

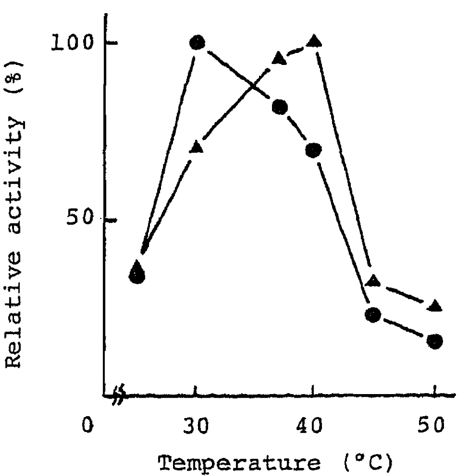


Fig. 6. Effect of Incubation Temperature on WIG Production

Each reaction mixture was incubated for 96 h at 25, 30, 37, 40, 45 and 50 °C. The pH's of the reaction mixtures were 5.5 (▲) and 7.5 (●). The degrees of relative activity are expressed as a percentage of the activity at 40 °C (pH 5.5) or 30 °C (pH 7.5). Each point represents the mean of three determinations.

7.0, yet each showed a distinct optimum and the appearance of WIG produced by each component was different.[6] Therefore, when the production of WIG was carried out by using a mixture of several components, such as cell-free culture fluid used in this experiment, the structure of WIG produced at each pH was expected to be different. However, the methylation analysis in this experiment showed that the structures of WIG produced at different pH values in the range 5.0 to 8.0 were the same, contrary to expectation. Moreover, the adherence of the WIG to the glass surfaces of the vessel was similar at all pH's.

Effect of Incubation Temperature on WIG Production——In Fig. 6, the effect of incubation temperature on WIG production is shown. When the pH was adjusted to 7.5, the amount of WIG produced was the largest at 30 °C. On the other hand, when the pH was not regulated, *i.e.*, pH 5.5, the amount of WIG produced was the largest at 40 °C. Thus, the effect of incubation temperature on the structure of WIG was investigated. The results at pH 7.5 are summarized in Table III; it was found that the ratios of the methyl *O*-methyl-D-glucopyranosides of WIG produced at 25, 30, 37, 40 °C were the same. However, those produced at 45 and 50 °C were very different from those produced at 25 to 40 °C. The WIG produced at 25 to 40 °C contained about 20% of 2,3,4-tri-*O*-methyl-D-glucopyranoside, *i.e.*, α-1,6 linkages, but those produced at 45 and 50 °C contained only 6 to 7% of α-1,6 linkages. Therefore, the activity and/or the stability of the enzyme component which took part in the synthesis of α-1,6 linkages might be low at above 45 °C. Moreover, it was observed that the WIG produced at 45 to 50 °C showed scarcely any adhesive property. Ebisu and Misaki and Koga and Inoue have reported that the adhesive property was attributable to α-1,6 linked chains.[4,19] Therefore, the results obtained in this experiment are consistent with theirs.

Table III. Effect of Incubation Temperature on WIG Structure

Temperature (°C)	Methyl *O*-methyl-D-glucopyranosides			
	2,3,4,6-Tetra-	2,4,6-Tri-	2,3,4-Tri-	2,4-Di-
25	11±1.5	57±2.6	21±2.1	11±1.7
30	12±1.5	57±0.6	20±2.1	11±0.6
37	15±1.5	54±1.2	20±0.6	11±0.6
40	10±1.2	57±1.5	23±0	10±1.0
45	8±1.0	83±1.0	6±1.2	3±0.6
50	5±2.1	85±1.2	7±1.0	3±1.0

Each value represents the mean molar percent±S.D. of three determinations. Each WIG was produced at pH 7.5.

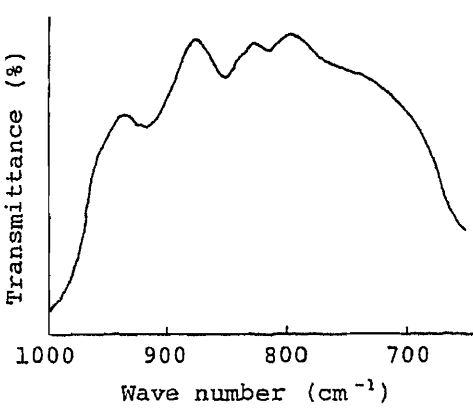


Fig. 7. IR Spectrum of WIG

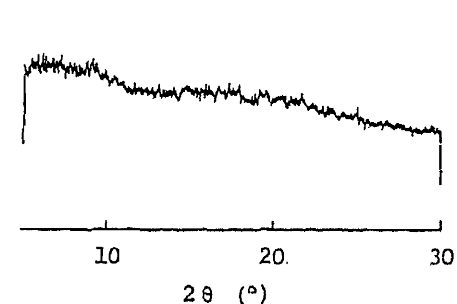
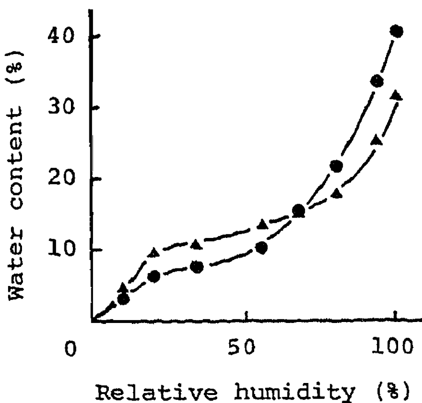


Fig. 8. Powder X-Ray Diffraction Pattern of WIG

In this experiment, the conditions of WIG production were investigated in order to improve tha yield of WIG, but the results are not yet adequate from the viewpoint of industrial production. Other culture conditions need to be investigated in order to further improve the yield of WIG. It was reported that the growth of several strains of *S. mutans* was faster chemically defined medium than in organic medium.[20] Accordingly, we investigated the utility of a chemically defined medium, which was a modification of the PD medium described by Ikeda *et al.*,[21] and was partially defined. Though the yield was improved somewhat, the WIG had a higher content, *i.e.*, about 50%, of α-1,6 linkage and showed a more adhesive character. WIG of this type might be worthy of further investigation. On the other hand Schachtele *et al.*[22] and Robyt and Corrigan[23] have reported that the addition of an exogenous primer such as dextran stimulated the activity of GTase, and Wittenberger *et al.*[24] and Umesaki *et al.*[25] have reported that the addition of Tween 80 stimulated the activity and/or the secretion of GTase. In addition, an increased yield of WIG by control of the salt concentration or the conditions of culture has been reported by Mukasa *et al.*[26] and Kenny and Cole,[27] respectively. Therefore, further examination of the conditions of the culture should lead to further improvement of the yield of WIG.

Physicochemical Properties of WIG

(IR) Spectrum——The IR spectrum of WIG over the range of 650—1000 cm^{-1} is shown in Fig. 7. The spectrum, with absorption bands assigned to α-1,3 linkages (815 and 850 cm^{-1}) and α-1,6 linkages (915 cm^{-1}), resembled that of WIG produced by *S. mutans* Ingbritt A or α-



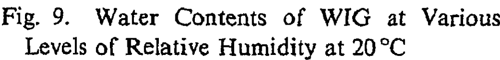


Fig. 9. Water Contents of WIG at Various Levels of Relative Humidity at 20 °C

●, water-insoluble glucan; ▲, corn-starch. Relative humidity was controlled by the use of various saturated salt solutions. Each point represents the mean of three determinations.

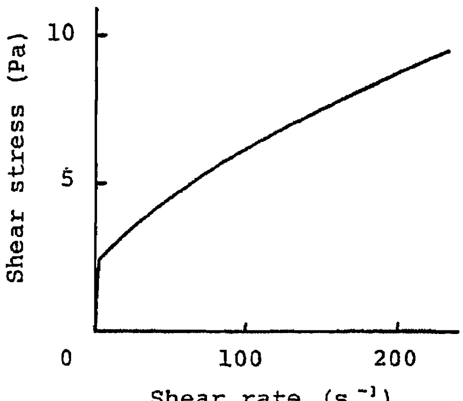


Fig. 10. Flow Curve of WIG Dispersion

The concentration of WIG was 6% and the temperature was 20 °C. The flow curve showed good reproducibility.

1,3 linkage-rich glucan extracted from *Polyporus betulinus*.[3] This result also shows this WIG to be exclusively α-1,3 linked.

Crystallinity——The powder X-ray diffraction pattern of WIG is shown in Fig. 8. WIG had no crystallinity, since no diffraction peak was ovserved in the pattern. Therefore, as reported for other polymers such as polyvinylpyrrolidone,[28] coprecipitates of poorly water-soluble drugs with WIG may be useful to improve the solubility of the drugs. A study on this subject is under consideration.

Hygroscopicity——The relationship between water content and relative humidity is shown in Fig. 9. For comparison, the relationship for corn starch is also shown in the same figure. The relationship for WIG showed a sigmoid-type pattern characteristic of water-insoluble polymers. The hygroscopicity of WIG was less than that of corn starch at under 70% relative humidity.

Flow Characteristic——The flow curve of WIG dispersion is shown in Fig. 10. The curve showed non-Newtonian and shear-thinning behavior. In addition, a yield point was observed. It has been suggested that a pseudoplastic vehicle is better as a suspending agent than a Newtonian vehicle.[29] Therefore, the application of WIG dispersion as a suspending agent may be useful. A rheological study of WIG is under consideration and will be reported elsewhere.

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Effect of Ultrasonic Irradiation on the Dissociation of Antigen–Antibody Complexes. Application to Homogeneous Enzyme Immunoassay

MAKOTO HAGA,*[,a] TAKESADA SHIMURA,[a] TOHRU NAKAMURA,[a] YURIKO KATO[a] and YASUO SUZUKI[b]

Faculty of Pharmaceutical Sciences, Science University of Tokyo,[a] Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan and Pharmaceutical Institute, Tohoku University,[b] Aobayama, Sendai 980, Japan

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The effect of ultrasonic irradiation on the dissociation of antigen–antibody complexes was studied. Among three frequencies tested, 28 kHz ultrasound gave a relatively high percent dissociation with the lowest decrease of immune reactivity. When dissolved antigen–antibody complexes were exposed to the ultrasonic irradiation no dissociation was observed, while antigen–antibody complexes were significantly dissociated by ultrasound when the antibodies had been immobilized on CH-Sepharose 4B. The immune reactivity of immobilized antibody decreased by 8% during irradiation with 28 kHz ultrasound (0.83 W/cm^2) for 20 min but no further decrease was observed after another 70 min of sonication. No degradation of antibody was observed after exposure to the ultrasound for 120 min. The dissociating effect of the ultrasound was applied to a homogeneous enzyme immunoassay system with the use of EMIT digoxin assay and anti-digoxin antibody-immobilized CH-Sepharose 4B gel. It was found that the change of absorbance at 340 nm increased with the concentration of non-labeled antigen in the presence of ultrasound. The feasibility of repeated application of immobilized antibodies in conjunction with ultrasonic dissociation as an immunosensor system is discussed.

Keywords——ultrasonic irradiation; dissociation; antigen–antibody complex; immobilized antibody; enzyme immunoassay

Much effort has been exerted during the last 10 years to develop high sensitive, specific, convenient and rapid enzyme immunoassay systems. The recent application of enzyme immunoelectrodes which convert chemical changes directly into electrical signals (*i.e.* enzyme immunosensor,[1–3] drugsensor,[4,5] and amperometric homogeneous immunoassay[6,7]) for the analysis of clinically and physiologically important substances in biological fluids has resulted in specific and simple assays. However, it is difficult to utilize this method for *in vivo* measurement. In order to measure drug concentration continuously with the use of an enzyme immunosensor, it is necessary either to supply antibodies from outside the system or to use the same antibodies repeatedly. In *in vivo* measurement it appears difficult to supply new antibodies to the sensor system. Thus, we have attempted to utilize ultrasonic irradiation to dissociate antigen–antibody complexes.

It has been demonstrated that mass transport of the substrate or product in an immobilized enzyme system is accelerated in the presence of ultrasonic irradiation (frequencies greater than 20 kHz).[8–10] Chetverikova *et al.*[11] have shown that therapeutic intensities of continuous wave of 0.88 MHz ultrasound had no detectable direct effect on the reaction rates of purified enzymes. The effect of 1.2 MHz ultrasound on deoxyribonucleic acid (DNA) synthesis has also been reported by Kondo and Yoshii.[12] Recently, it has been demonstrated that the use of 50 kHz ultrasound to enhance mass transport across liquid/solid interfaces can

dramatically accelerate antigen binding to immobilized antibodies.[13] Until now, however, no report has dealt with the effect of ultrasonic irradiation on the immune reactivity of antibodies, especially on the dissociation of antigen–antibody complexes. In this report we describe the effect of ultrasonic irradiation on the dissociation of antigen–antibody complexes and its application to a homogeneous enzyme immunoassay with the use of EMIT digoxin assay.

Materials and Methods

Materials——Digoxin and glucose-6-phosphate dehydrogenase (G-6-PDH) from *Leuconostoc mesenteroides* were obtained from Sigma Chemicals Co. (St. Louis, MO), and bovine serum albumin (BSA) was from Armour Pharmaceutical Co. (Phoenix, Ariz.). Sodium laurylsulfate (SDS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), acrylamide, *N,N'*-methylenebisacrylamide, Coomassie brilliant blue and diethylaminoethyl (DEAE)-cellulose were purchased from Nakarai Chemicals, Ltd. (Kyoto). Complete Freund's adjuvant was a product of Iatron Laboratories (Tokyo) and bromophenol blue was from Kanto Chemicals Co. (Tokyo). Sepharose 4B and Sephadex G-50 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and [^{3}H(G)]-digoxin (22 Ci/mmol) from NEN Research Products (Boston, MA). Soluene-350 and Scintisol EX-H were obtained from Pachard-Becker B.V. Chemical Operations (The Netherlands) and Dojindo Laboratories (Kumamoto, Japan), respectively. EMIT digoxin assay batch reagents were obtained from Syva (Palo Alto, CA). Other chemicals used were commercial products of high purity and all operations utilized freshly redistilled water.

Apparatus——Three types of sonicator (Sun Electronics, Tokyo, SC-20A; Kaijo Electric, Tokyo, TA-4021; Ito Ultrasonic, Tokyo, KUS-2) were used to provide ultrasound at 28 kHz, 420 kHz and 1 MHz, respectively. Input wattage was measured with a high-frequency wattmeter (Toa Electronics Ltd., OP-1RA). Sound pressure and frequency were measured with an NTK ultrasonic meter (UTK-30). Samples were sonicated in a bath thermostated at 5—6 °C with a Lauda K2RD temperature controller.

Preparation of Anti-digoxin Immunoglobulin G (IgG)——Digoxin–BSA conjugates were prepared according to the method of Butler and Chen.[14] Digoxin (50 mg) was dissolved in 12.5 ml of absolute ethanol–dioxane mixture (ethanol : dioxane = 4 : 1) and 7.5 ml of 0.05 M sodium metaperiodate was added. The mixture was allowed to stand in the dark at 4 °C for 20 min. The reaction was terminated by adding 0.68 ml of 1 M ethylene glycol. BSA solution (2.5 ml; concentration, 8 mg/ml) was added and the pH of the mixture was adjusted to 9.3 with 5% K_2CO_3. The mixture was incubated at room temperature for 1 h, then 150 mg of sodium borohydride was added, and the mixture was allowed to stand for 24 h. The pH of the solution was adjusted to 5.5 by adding about 5.4 ml of 1 M formic acid. After 1 h, 1.5 ml of 1 M NH_4OH was added to raise the pH to 8.5 and the mixture was dialyzed overnight against distilled water. The pH was lowered to 4.8 by addition of 0.1 N HCl. The suspension was allowed to stand at 4 °C for 4 h, then centrifuged for 1 h at 1000 *g*. The precipitate was dissolved in 0.15 M carbonate buffer and this solution was dialyzed for 5 d against distilled water, lyophilized and stored at −20 °C. The digoxin–BSA conjugate in 83% H_2SO_4 had absorption maxima at 388 and 465 nm. From the absorbance at 388 nm, we estimated that 4.6 digoxin residues were conjugated per BSA molecule. Two female New Zealand White rabbits were immunized with the digoxin–BSA conjugate. A saline solution of the conjugate (1 mg/ml) was mixed with 1 ml of complete Freund's adjuvant and 1.5 ml of this suspension was injected subcutaneously into the pads and at sites on the back. Rabbits were immunized once a week for the first three weeks and thereafter once every 2 weeks. Antisera were collected separately from the two rabbits by 6 months after the initial immunization. The IgG fraction of anti-digoxin antiserum was separated by precipitation with 40% saturated $(NH_4)_2SO_4$ followed by purification on a DEAE cellulose column (2 × 45 cm) with 0.1 M Tris–phosphate buffer (pH 8.5). The nonadsorbed fractions were pooled, lyophilized and stored at −20 °C.

Immobilization of Antibodies——The antibodies were immobilized on CH-Sepharose 4B according to the instructions of Pharmacia. CH-Sepharose 4B (2.5 g) was washed with 0.5 M NaCl solution, then equilibrated with 0.1 M phosphate–HCl buffer (pH 5.5). The gel was incubated with 150 mg of EDC in 5 ml of 0.1 M phosphate–HCl buffer (pH 5.5) at room temperature for 30 min, then 5 mg of anti-digoxin IgG dissolved in 5 ml of the same buffer was added. The mixture was stirred overnight with an end-over-end type rotator at 25 rpm. After the reaction had been completed, the gel was washed thoroughly with 10 mM phosphate buffer (pH 7.0) containing 0.1% sodium azide and stored at 4 °C. The amount of immobilized antibody on the CH-Sepharose 4B was determined by an radio immunoassay (RIA) using ^{3}H-digoxin. The anti-digoxin IgG-bound CH-Sepharose 4B was incubated overnight with excess ^{3}H-digoxin at 4 °C and washed thoroughly with 0.2 M Gly–HCl buffer (pH 3.3) and 10 mM phosphate buffer (pH 7.0) alternately. The gel (0.5 ml) was poured into a glass tube to make a column (0.5 × 2.55 cm). The ^{3}H-digoxin was eluted with 0.2 M Gly–HCl buffer (pH 2.2) and measured in a liquid scintillation counter (Aloka LSC-673).

Dissociation of Antigen–Antibody Complexes——A digoxin solution (2.5 mg/650 μl of a 4 : 1 mixture of absolute ethanol and dioxane) was incubated with 3 mg of anti-digoxin IgG dissolved in 1.5 ml of 10 mM phosphate buffer (pH 7.0) at 4 °C overnight. The antigen–antibody complexes produced were separated from free antigen and antibody by gel filtration on a Sephadex G-50 column with 10 mM phosphate buffer (pH 7.0). The complex fraction (2 ml,

concentration of IgG was 2.40×10^{-10} M) was mixed with 50 μl of 4.60×10^{-7} M ^{3}H-digoxin (about 1.12×10^6 dpm) in a vial and the mixture was sonicated for 5—20 min. The mixture was allowed to stand for 30 min and then gel-filtered on a Sephadex G-50 column (1.2×20 cm) with 10 mM phosphate buffer (pH 7.0). The fractions of IgG were collected and the radioactivity was measured to calculate the percentage of dissociation. In the case of dissociation using immobilized antibody, 0.5 ml of IgG-bound CH-Sepharose 4B was incubated with excess ^{3}H-digoxin at 4 °C overnight. The gel was washed completely with 0.2 M Gly–HCl buffer (pH 3.2) and 10 mM phosphate buffer (pH 7.0) alternately, then 2 ml of 10 mM phosphate buffer (pH 7.0) was added. The gel was sonicated for 3—20 min, then centrifuged immediately (3000 rpm, 5 min) and the radioactivity in the supernatant (200 μl) was measured in a liquid scintillation counter.

Detection of the Change of Immune Reactivity——Anti-digoxin IgG (1.2 mg) was dissolved in 50 ml of 0.1 M Tris-HCl buffer (pH 7.0) and 2.0 ml of the IgG solution was sonicated for 5—90 min in a vial. The sonicated IgG solution (100 μl), ^{3}H-digoxin (50 μl, about 50000 dpm), normal rabbit serum (100 μl) and the buffer (250 μl) were mixed in a vial and allowed to stand at 4 °C overnight. Then 500 μl of 50% saturated $(NH_4)_2SO_4$ was added, and the suspension formed was centrifuged at 3000 rpm for 30 min. The supernatant was carefully removed and 0.5 ml of Soluene-350 was added. The mixture was incubated at 50 °C for 2 h, and 6 ml of Scintisol was added. The radioactivity of antibody-bound ^{3}H-digoxin was measured in a liquid scintillation counter. To detect the change of the immune reactivity of immobilized antibody, 0.5 ml of IgG-bound CH-Sepharose 4B was sonicated for 5—70 min and incubated with excess ^{3}H-digoxin at 4 °C overnight. The gel was washed completely with 0.2 M Gly–HCl buffer (pH 3.2) and 10 mM phosphate buffer (pH 7.0) alternately. The ^{3}H-digoxin was eluted with 0.2 M Gly–HCl buffer (pH 2.2) and the radioactivity was measured.

Electrophoresis——Electrophoresis was carried out according to the method of Laemmli.[15] The stacking gel of 8% acrylamide contained 0.125 M Tris–glycine and 0.1% SDS. The electrode buffer contained 0.125 M Tris–glycine and 0.1% SDS. Samples (0.2—0.3 ml; protein concentration, 1—2 mg/ml) were prepared with 10 mM Tris–HCl buffer (pH 6.8) containing 1% bromophenol blue as the dye. The proteins were completely dissociated by immersing the samples for 2 min in boiling water. Electrophoresis was carried out with a constant current (10 mA) until the marker reached the bottom of the gel (about 5 h). The proteins were fixed in the gel with 12.5% trichloroacetic acid for 15 min and stained at 37 °C for 1 h with 0.05% Coomassie brilliant blue R-250 solution. Then, the gels were destained in a mixed solution of 25% ethanol and 8% acetic acid.

Application to EMIT Assay——The enzyme-labeled digoxin (reagent B of the EMIT kit) and the immobilized anti-digoxin IgG on CH-Sepharose 4B were used for digoxin assay. The gel (3 ml; amount of immobilized IgG, 1.74 pmol) equilibrated in buffer A of the EMIT kit (0.055 M Tris–HCl containing 0.005% Tween 20; pH 7.4) was mixed with 500 μl of reagent B and incubated at room temperature for 15 min with the use of an end-over-end type rotator. Then 0.5 ml of the calibrator and 100 μl of 0.5 N NaOH were added to the gel and the mixture was sonicated at 30 °C for a certain period of time between zero and 20 min. Immediately after the sonication, 1.7 ml of substrate solution (18.5 mg of G-6-P, 27.5 mg of nicotinamide adenine dinucleotide (NAD)/12.5 ml of buffer A) was added to the gel. The mixture was incubated at 30 °C for a period of 30 min minus the sonication time, cooled to 0 °C and centrifuged at 4 °C. The absorbance at 340 nm of the supernatant (with or without sonication, A_2 or A_1) was measured and the difference ΔA_{340} ($= A_2 - A_1$) was calculated. In the feasibility assessment of repeated assay with the use of dissociation of antigen–antibody complexes by ultrasonic irradiation, the following procedures were carried out. The gel (5 ml; amount of immobilized IgG, 0.39 pmol/ml of gel) equilibrated in buffer A was mixed with 1 ml of reagent B and preincubated for 15 min with the use of an end-over-end type rotator. The mixture was centrifuged (3400 rpm, 5 min) and the supernatant was removed. Then, 500 μl of calibrator and 150 μl of 0.5 N NaOH were added to the gel. The mixture was sonicated at 30 °C for 10 min, then 1.7 ml of substrate solution was added, and the whole was incubated at 30 °C for another 20 min. The gel mixture was cooled to 0 °C and centrifuged at 4 °C. The supernatant was transferred to a cuvette and the change of the absorbance was measured. The rate of change of absorbance (ΔA_1/min) was calculated. The supernatant (about 1.6 ml) was dialyzed against buffer A for 6 h to remove free nonlabeled digoxin. Then, 500 μl of calibrator and 150 μl of 0.5 N NaOH were added to the inner solution, mixed and brought back to the gel. The mixture was sonicated again at 30 °C for 10 min, then 1.7 ml of substrate solution was added and the mixture was incubated at 30 °C for another 20 min. The gel mixture was cooled to 0 °C and centrifuged at 4 °C. The supernatant was transferred to a cuvette and the change of absorbance was measured. The rate of change of absorbance (ΔA_2/min) was calculated.

Effect of Sonication on G-6-PDH Activity——A G-6-PDH solution (2U/1 ml) and a substrate solution which contained G-6-P ($1.06 \times 10^{-3} - 1.06 \times 10^{-4}$ mol/2 ml) and NAD (6.14 μmol) in buffer A were prepared. Either G-6-PDH solution or substrate solution was sonicated in a test tube at 5—6 °C for 10 min. The absorbance at 340 nm (A_I) was measured immediately after addition of 10 μl of the G-6-PDH solution (0.02 U) to 2 ml of the substrate. After incubation at 30 °C for 20 min the absorbance at 340 nm (A_F) was measured again and the difference between A_F and A_I was calculated.

Results

Effect of Frequency and Depth of Water

The effects of frequency of ultrasound on the dissociation of antigen–antibody complexes and on the immune reactivity were studied by using three types of sonicator operating at 28 kHz, 420 kHz and 1 MHz. Irradiation at 1 MHz (5 W/cm^2) for 10 min dissociated about 12% of the antigen from immobilized antibody. On the other hand, irradiation at 420 and 28 kHz (0.83 W/cm^2) for 10 min dissociated about 15% of the antigen. However, the immune reactivity decreased significantly when 420 kHz ultrasound was used. The temperature in the vial which contained CH-Sepharose 4B gel did not rise above 5 °C during sonication for 10 min. Thus, we used 28 kHz ultrasound in the subsequent investigation.

In bath-type sonication, the ultrasonic intensity is significantly affected by the depth of water in the bath. The relations between the input wattage (W/cm^2), sound pressure (μPa), frequency (kHz) and the depth of water (cm) were studied using a 28 kHz sonicator (Sun Electronics, SC-20A). The largest input wattage (1.31 W/cm^2) was obtained at 4 cm (1.04 W/cm^2 at 6.5 cm and 0.83 W/cm^2 at 6 cm). The intensity of sound pressure increased with depth in the order of 6, 8, 10, and 4 cm. The frequency was stable at 6, 8 and 10 cm but very unstable at 4 and 6.5 cm. Figure 1 shows the effect of depth of water on the percent dissociation of immobilized antigen–antibody complexes. Maximal values of percent dissociation were obtained at 4, 6 and 8 cm. The effect of depth of water on the immune reactivity of dissolved antibody is shown in Fig. 2. As shown in Fig. 2 the decrease of immune reactivity was 8, 14.6 and 22.2% at 6, 4 and 8 cm, respectively, On the basis of these findings, we set the depth of water at 6 cm.

Effect of Sonication on the Dissociation of Antigen–Antibody Complexes

The effect of ultrasound irradiation (28 kHz, 0.83 W/cm^2) on the dissociation of antigen–

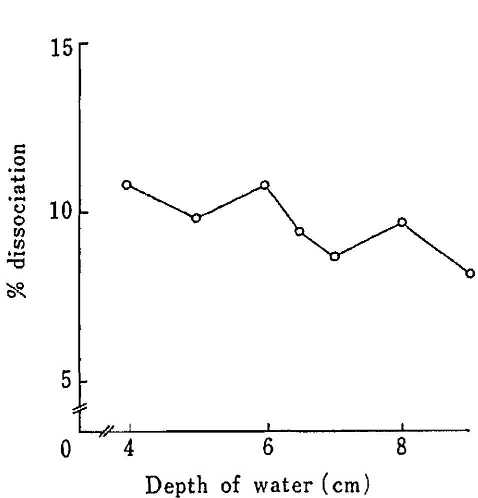

Fig. 1. Effect of the Depth of Water on the Percent Dissociation of Immobilized Antigen–Antibody Complexes

The anti-digoxin IgG immobilized on CH-Sepharose 4B (0.5 ml; amount of immobilized IgG, 0.39 pmol/ml of gel) was incubated with excess ^{3}H-digoxin at 4 °C overnight and the gel was washed completely. The antigen–antibody complexes were sonicated (28 kHz, 0.83 W/cm^2) in the indicated depth of water for 10 min.

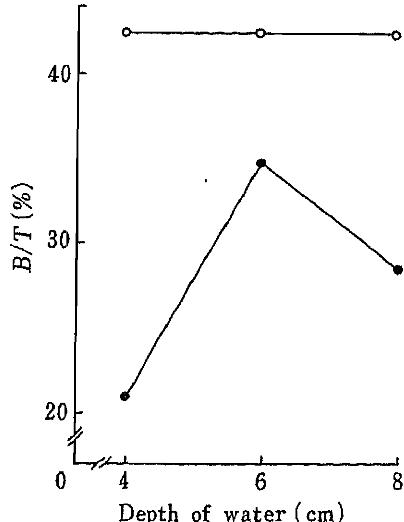

Fig. 2. Effect of the Depth of Water on the Immune Reactivity of Dissolved Antibody

A solution of anti-digoxin IgG (2 ml) was sonicated (28 kHz, 0.83 W/cm^2) for 10 min (●) and the radioactivity of the solution (100 μl) was measured. The decrease of immune reactivity was estimated from the change of B/T (B, radioactivity of bound fraction; T, total radioactivity). Control (○) was unsonicated.

antibody complexes was first investigated by using dissolved antibodies. As shown in Fig. 3, no significant increase in percent dissociation was observed. In contrast, the antigen–antibody complexes were significantly dissociated when the antibodies had been immobilized on CH-Sepharose 4B. The percent dissociation increased with both irradiation time and input wattage.

Effect of Sonication on Immune Reactivity

To test the effect of ultrasound on the immune reactivity, we examined the change of B/T (B, radioactivity of bound fraction; T, total radioactivity) in the presence of sonication using ^{3}H-labeled digoxin and immobilized digoxin-specific antibodies. Figure 4 shows the effect of sonication time on the immune reactivity of immobilized antibodies. The amount of immunologically intact antibodies decreased by about 8% during the initial 20 min, then leveled off and did not change for 70 min. The percent dissociation of antigen–antibody complexes exposed to 0.83 W/cm^2 of ultrasound was 22% at 20 min (Fig. 3), whereas the decrease of immune reactivity of immobilized antibody exposed to 0.83 W/cm^2 of ultrasound for 20 min was 8% (Fig. 4). From these findings it was confirmed that, although the immune reactivity of immobilized antibody is impaired to some extent in the presence of ultrasound, the antibody is still active even after the dissociation. The change in molecular weight of antibody in the presence of ultrasound was also investigated by SDS–polyacrylamide slab gel electrophoresis. No degradation of dissolved antibody was observed after exposure to 0.83 W/cm^2 of ultrasound for times varying from 30 to 120 min.

Assessment of Assay Feasibility

We tried to apply the dissociating effect of ultrasound to an enzyme immunoassay system, by choosing a homogeneous EMIT assay. The effect of ultrasound on the enzyme activity of G-6-PDH was first investigated. Figure 5 shows Lineweaver–Burk plots of G-6-

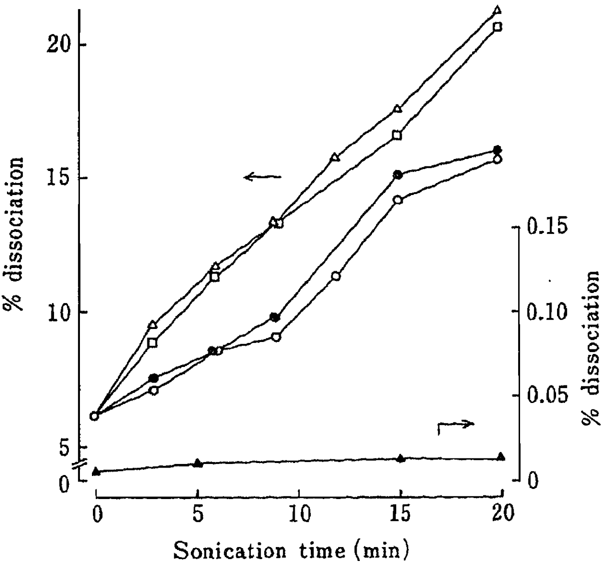

Fig. 3. Effect of Sonication Time on the Percent Dissociation of Dissolved and Immobilized Antigen–Antibody Complexes

Solutions of dissolved antigen–antibody complexes (2 ml, ▲) and the immobilized antigen–antibody complexes (0.5 ml of gel) were incubated with ^{3}H-digoxin and sonicated (28 kHz; depth of water 6 cm) for the indicated time. Input wattage was as follows: 18 W (○); 26 W, (●); 44 W, (□); 65 W (△). The points represent averages of two measurements.

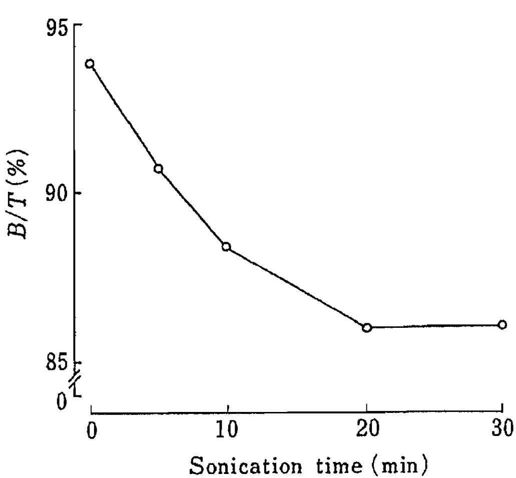

Fig. 4. Effect of Sonication Time on the Immune Reactivity of Immobilized Antibody

Anti-digoxin IgG immobilized on CH-Sepharose 4B (0.5 ml) was sonicated (28 kHz; 0.83 W/cm^2; depth of water, 6 cm) for the indicated time. The gel was incubated with ^{3}H-digoxin and the amount of antibody was measured by RIA. The points represent averages of two measurements.

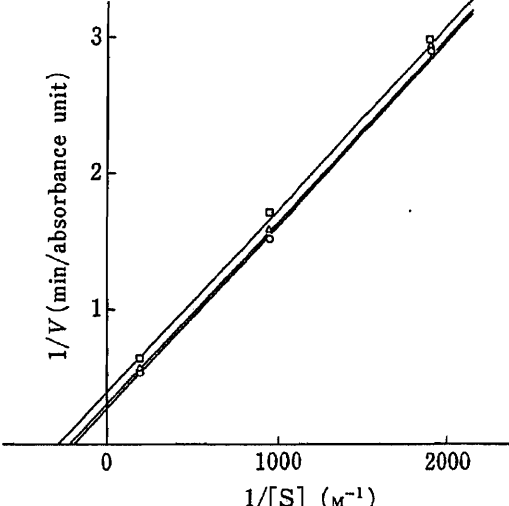

Fig. 5. Lineweaver–Burk Plots of G-6-PDH Activity

Either G-6-PDH solution (2 U/ml, □) or substrate solution (G-6-P and NAD, △) was sonicated for 10 min. The absorbance at 340 nm was measured as described in Materials and Methods. The control (○) was unsonicated.

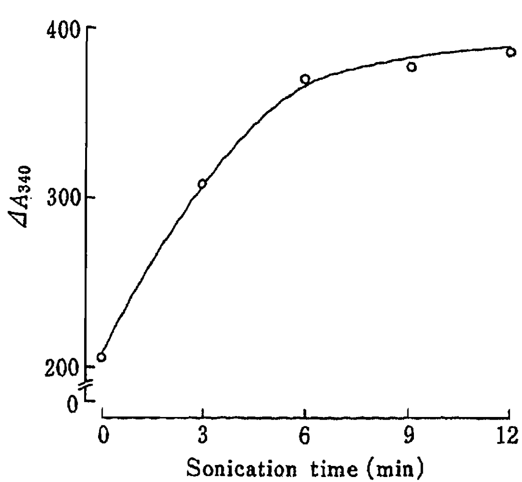

Fig. 6. Effect of Sonication Time on ΔA_{340}

Anti-digoxin IgG (1.74 pmol) immobilized on CH-Sepharose 4B (3 ml) was incubated with G-6-PDH-labeled digoxin (0.5 ml of reagent B). The antigen–antibody complexes were sonicated (28 kHz; 0.83 W/cm^2; depth of water, 6 cm) with 0.5 ml of digoxin solution (3.2×10^{-13} mol) for the indicated time. ΔA_{340} was measured as described in Materials and Methods. The points represent averages of two measurements.

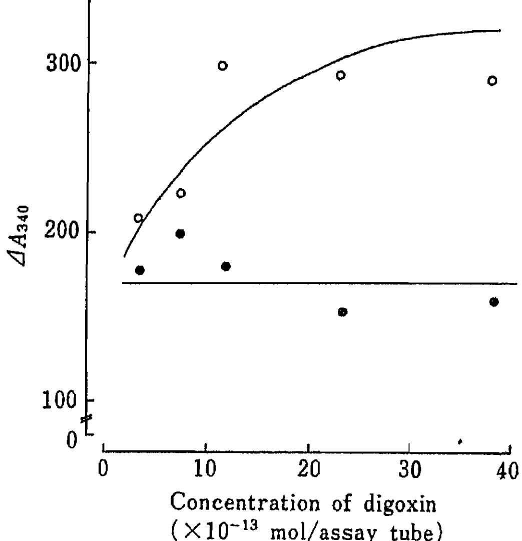

Fig. 7. Calibration Curve for Digoxin

Anti-digoxin IgG immobilized on CH-Sepharose 4B (3 ml) was incubated with G-6-PDH-labeled digoxin (0.5 ml of reagent B). The antigen–antibody complexes were sonicated (28 kHz; 0.83 W/cm^2; depth of water, 6 cm) with the indicated concentration of digoxin for 10 min (○). The control (●) was unsonicated. ΔA_{340} was measured as described in Materials and Methods. The points represent averages of two measurements.

PDH activity when G-6-PDH or substrate solution was exposed to 0.83 W/cm^2 of ultrasound for 10 min. The K_m values calculated from the slopes were 5.02×10^{-3}M for the control, 5.61×10^{-3}M for sonicated G-6-PDH and 3.95×10^{-3}M for sonicated substrate and NAD. The V_{max} values were 0.179 (absorbance/min) for the control, 0.200 for sonicated G-6-PDH and 0.131 for sonicated substrate and NAD. These findings suggest that there are no significant effects of ultrasound on the enzyme activity and substrate or NAD.

Figure 6 shows the effect of sonication time on ΔA_{340} values. The ΔA_{340} increased with increasing sonication time and leveled off at about 10 min. The value of ΔA_{340} without sonication was about 0.2, which was attributed to the excess amount of enzyme-labeled antigen existing in the solution. Thus, the sonication time was set at 10 min in the following

experiment. Figure 7 shows the change of ΔA_{340} with various concentrations of digoxin in the presence or absence of ultrasound. In the absence of ultrasound, radiated ΔA_{340} was constant. This indicates that the amount of free enzyme-labeled digoxin remained constant irrespective of the concentration of non-labeled digoxin. On the other hand, in the presence of ultrasound ΔA_{340} increased with increasing concentration of non-labeled digoxin. It is reasonable to consider that the amount of free enzyme-labeled digoxin increased as a result of the competitive reaction between nonlabeled digoxin and enzyme-labeled digoxin, which was dissociated from the antigen–antibody complex by ultrasonic irradiation. The results in Fig. 7 provide the basis for an enzyme immunoassay which utilizes dissociation of antigen–antibody complexes by sonication.

Discussion

In the present work we studied the effect of ultrasound on the dissociation of antigen–antibody complexes. When dissolved antigen-antibody complexes were exposed to ultrasound, no dissociation was observed, while antigen–antibody complexes immobilized on CH-Sepharose 4B were significantly dissociated by the exposure. The percent dissociation increased with irradiation time and input wattage of the ultrasound. The oscillation of cavitation bubbles is known to cause hydrodynamic shearing stress.[16] It is considered that the antigen–antibody complexes were dissociated by this shearing stress only when the antibodies were immobilized on CH-Sepharose 4B. Among the frequencies of ultrasound tested, 28 kHz gave a relatively high percent dissociation and the lowest decrease of immune reactivity. The immune reactivity of the immobilized antibody decreased by about 8% after 20 min of sonication but no further decrease was observed after another 70 min of sonication. No degradation of antibody was observed after exposure to the ultrasound (28 kHz, 0.83 W/cm^2) for 120 min. The dissociating effect of the ultrasound was applied to a homogeneous enzyme immunoassay system using the EMIT assay, and it was found that the increase of ΔA_{340} was parallel with that of the concentration of non-labeled antigen in the presence of ultrasound.

To investigate the possibility of repeated use of the same immobilized antibodies in the homogeneous enzyme immunoassay with the use of ultrasound dissociation, we tried to calculate a standard curve for unlabeled digoxin to be measured. In the following consideration, it is assumed that the amount of enzyme-labeled antigen is twice the amount of antibody (N) since IgG is used as antibody, and that there is no difference between enzyme-labeled antigen and non-labeled antigen with respect to diffusion and binding processes. If the amount of enzyme-labeled antigen in the system is constant at every measurement ($2N$) and free non-labeled antigen is eliminated by dialysis after each measurement, the following equations may be written for the amount of bound enzyme-labeled antigen (A_n) and the amount of bound non-labeled antigen (B_n) at the n-th measurement, respectively.

$$A_n = \frac{NX(A_{n-1}X + 100B_{n-1})}{50[20NX + 100(B_{n-1} + M_{nm})]} + \frac{A_{n-1}(100 - X)}{100} \quad (1)$$

$$B_n = \frac{NX(B_{n-1} + 100M_{nm})}{50[20NX + 100(B_{n-1} + M_{nm})]} + \frac{B_{n-1}(100 - X)}{100} \quad (2)$$

where X is the percent dissociation and M_{nm} is the amount of nonlabeled antigen to be assayed. Thus A_n can be calculated by using the amount of bound enzyme-labeled antigen (A_{n-1}) and the amount of bound non-labeled antigen (B_{n-1}) from the $n-1$th measurement. The amount of free enzyme-labeled antigen at the n-th measurement is:

$$F_n = 2N - A_n (= B_n) \quad (3)$$

Assuming that one antigen molecule is conjugated per one enzyme molecule, then F_n is equal to the amount of enzyme (E_n) in the solution. Since the enzyme is deactivated when the enzyme-labeled antigen binds with antibody, the change of absorbance (ΔA_n, milliabsorbance unit/min) is proportional to the amount of free enzyme-labeled antigen:

$$\Delta A_n = K_1 E_n = K_1 B_n \tag{4}$$

where K_1 is a proportionality constant. Therefore, if K_1 is known, B_{n-1} may be calculated from the value of A_{n-1}, and B_n from the value of ΔA_n by using Eq. 4. Accordingly, M_{nm} can be estimated from Eq. 2. In the EMIT assay the relation between the change of absorbance at 340 nm in 30 min ($\Delta A/30$, milliabsorbance unit/30 min) and the amount of non-labeled antigen (M) is expressed as follows:

$$\Delta A/30 = K_2[Y-(2N-M)] \tag{5}$$

where N is the amount of antibody ($2N > M$), Y is the amount of enzyme-labeled antigen and K_2 is a proportionality constant. The term $Y-(2N-M)$ in Eq. 5 is equal to E. Rearrangement of Eq. 4 yields: $\Delta A/30 = K_2(Y-2N) + K_2 M$. Therefore, the proportionality constant K_2 can be readily calculated from the slope of the straight line plot of $\Delta A/30$ *versus* M. From the standard curve of the EMIT digoxin assay batch used in our experiment K_2 was calculated to be 1.95×10^{14} mol^{-1}, hence, K_1 was 6.51×10^{12} mol.$^{-1}$ Figure 8 shows observed values of ΔA_2 at indicated concentrations of digoxin when ΔA_1 was observed to be 0.20 before each measurement. Calculated curves for ΔA_2 assuming that X is equal to 14 or 25 are also shown in Fig. 8, when 1.2×10^{-12} mol of antibody was used. These curves were generated using Eqs. 2 and 4. When it is assumed that X is equal to 25, the calculated curve is in approximate agreement with the observed values. However, the observed % dissociation was about 14 as shown in Fig. 3. This discrepancy may be explained as follows. In our assay system digoxin competes with enzyme-labeled digoxin for the binding site of the antibody during or after irradiation with ultrasound. Equations 1 and 2 are derived assuming that there is no differrence in the diffusion process between enzyme-labeled digoxin and non-labeled digoxin. However, it is considered that the diffusion coefficient of non-labeled digoxin is larger than that of enzyme-labeled digoxin[17] and non-labeled digoxin binds predominantly to immobilized antibody. Consequently, the amount of free enzyme-labeled digoxin which can be observed may exceed the calculated values. The other possible explanation for the discrepancy is that the enzyme G-6-PDH exists as a dimer.[18,19] In the case that immobilized antibodies are used, even if the enzyme-labeled antigen binds to the antibody, the other active site of the enzyme will not be inhibited because of the immobilization of the antibodies. In this case, a larger ΔA_n value may be observed than the calculated value. The number of antigen molecules conjugated to an enzyme molecule is another problem. If the number is not equal to one, then

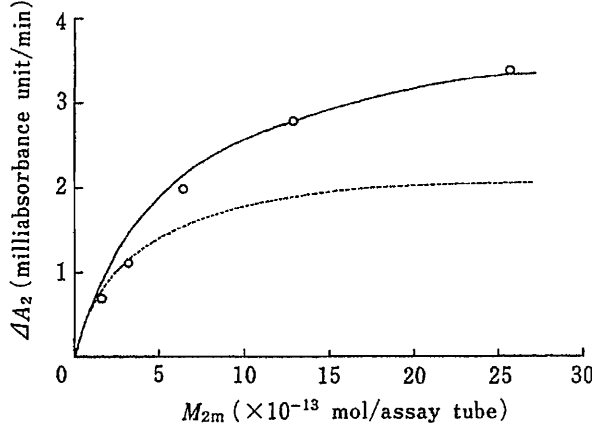

Fig. 8. Calculated Curves and Observed Values for ΔA_2/min

The ΔA_1 was observed to be 0.20 before each measurement of ΔA_2 at the indicated concentration of digoxin. Calculated values for ΔA_2 were generated using Eqs. 2 and 4 assuming that X is equal to 14 (dotted line) or 25 (solid line). ΔA_2/min was measured as described in Materials and Methods. The points represent averages of two measurements.

$F_n \neq E_n$ and the right side of Eq. 5 must be divided by this number. This is the case when dissolved antibodies are used. However, when antibodies are immobilized it is reasonable to consider that the relation between the number of antigen and antibody molecules is the same when the enzyme-labeled antigen (antigen/enzyme = 1) is used, since the antibody can not diffuse freely.

In this experiment we tested the dissociating procedure of antigen–antibody complexes only a few times. Further work remains to be done in order to measure *in vivo* drug concentration with this type of method. In particular, it is necessary to find a more suitable frequency at which a higher percent dissociation and a lower decrease of immune reactivity can be obtained. However, these results do demonstrate the feasibility of using an enzyme immunosensor *in vivo*. The dissociating effect of ultrasound may also be applicable in the area of affinity chromatography.

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Kinetics and Mechanism of the Acid–Base Equilibrium of Mexazolam and Comparison with Those of Other Commercial Benzodiazepinooxazole Drugs[1]

YUKIHISA KURONO,*[,a] KINYA KAMIYA,[a] TOMONARI KUWAYAMA,[b] YASUHIRO JINNO,[a] TAMOTSU YASHIRO,[a] and KEN IKEDA[a]

Faculty of Pharmaceutical Sciences, Nagoya City University,[a] 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan and Pharmacy, Tokai Teishin Hospital,[b] 2–17–5 Matsubara, Naka-ku, Nagoya 460, Japan

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The oxazolidine ring-opening and ring-closing reactions of mexazolam, cloxazolam, haloxazolam, and flutazolam were investigated by a pH-jump method, similarly to the case of oxazolam reported previously [Kurono *et al.*, *Chem. Pharm. Bull.*, **33**, 1633 (1985)]. Mexazolam exists essentially as a single isomer, either *cis* or *trans* (referring to the 3-methyl group and 11b-(2′-chlorophenyl) group), differently from the case of oxazolam (*cis* isomer/*trans* isomer ratio for oxazolam≒1 : 1). Over the pH range of 1—13, the pH–rate profiles show two step reactions. For interpretation of these profiles, we propose a reaction mechanism including an intermediate, detected by the kinetic method, between the iminium structure (oxazolidine ring-opened form) and the ring-closed form. These kinetic properties of mexazolam differ from those of other benzodiazepinooxazoles, and the difference is caused by the presence of the 3-methyl group rather than the 2′-chlorine atom. The intrinsic rate constants of mexazolam, its 2′-chlorine deficient analog (3-methyl compound), cloxazolam, haloxazolam, and flutazolam were determined according to the appropriate reaction schemes.

Keywords——mexazolam; cloxazolam; haloxazolam; flutazolam; benzodiazepinooxazole; oxazolidine ring-opening, -closing; kinetics; *cis–trans* isomer; acid–base equilibrium; pH–rate profile

In the previous paper[2] we reported the kinetics and mechanism of the oxazolidine ring-opening and ring-closing reactions (acid–base equilibrium) of oxazolam. Rate measurements using a stopped-flow spectrophotometer allowed us to distinguish between the *cis* and *trans* isomers (referring to the 2-methyl group and 11b-phenyl group). Mexazolam is also considered to possess *cis* and *trans* isomers (referring to the 3-methyl group and 11b-(2′-chlorophenyl) group). However, when the rates of the ring-opening and -closing reactions of mexazolam were examined, the kinetic behavior of mexazolam was found to be very different from that of oxazolam and other benzodiazepinooxazoles.

In this paper we describe at first the kinetic results for cloxazolam, haloxazolam, and flutazolam (commercial benzodiazepinooxazole drugs) for easy understanding of the difference in the reaction mechanism between mexazolam and other benzodiazepinooxazoles. Then we propose a reaction mechanism for the rate process of the acid–base equilibrium of mexazolam. The results for the 2′-chlorine deficient analog of mexazolam (3-methyl compound) synthesized are also presented. The chemical structures of the drugs used are shown in Chart 1.

	R_2	$R_{2'}$	R_3	R_7	R_{10}
cloxazolam	H	Cl	H	H	Cl
haloxazolam	H	F	H	H	Br
flutazolam	H	F	H	C_2H_4OH	Cl
mexazolam	H	Cl	CH_3	H	Cl
3-methyl compound	H	H	CH_3	H	Cl
oxazolam	CH_3	H	H	H	Cl

Chart 1

Experimental

Materials and Instruments——Mexazolam (lot No. 2), cloxazolam (lot No. 2), and haloxazolam (lot No. 6) were supplied by Sankyo Co., Ltd. Flutazolam (lot No. A 424490) was a gift from Mitsui Pharmaceutical Co., Ltd. All the drugs were used without further purification. The 3-methyl compound was synthesized by a procedure similar to those reported by Deriege *et al.*,[3] Miyadera *et al.*,[4] and Lemke and Hanze.[5] All other chemicals were purchased commercially and were of reagent grade.

Ultraviolet (UV) absorption spectroscopy was carried out with a Hitachi UV-124 spectrophotometer and a Shimadzu UV-260 spectrophotometer. The reaction rates were measured with a Hitachi UV-124 spectrophotometer. ^{1}H- and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were obtained with a JEOL JNM-FX 100 spectrometer at 100 and 25 MHz, respectively, using tetramethylsilane as an internal standard. A Hitachi-Horiba F-7_{LC} pH meter was used for pH measurement. An NEC microcomputer (PC-9801E) was employed for the calculation of rate constants and equilibrium constants and for the simulation of the pH–rate profiles.

Determination of Equilibrium Constant——The apparent equilibrium constants and dissociation constants of benzodiazepinooxazoles were determined by methods similar to those reported previously.[2,6]

Kinetic Procedures——The buffer systems were the same as those used in the previous studies.[2] The UV spectra of mexazolam at pH 3—5 were measured in 0.033 M glycine–HCl buffer and 0.017 M acetate buffer (one third or one sixth of the ordinary buffer concentrations) because 0.1 M glycine and 0.1 M acetate buffers show significant absorbances below 230 nm and disturb the spectra of mexazolam below 230 nm.

Rate measurements of the oxazolidine ring-opening and ring-closing were carried out by the pH-jump method as reported previously.[2] In addition to the stopped-flow method, the conventional UV method was also applied for the slow rate measurements of mexazolam and the 3-methyl compound. All the experiments except for the ^{1}H-NMR and ^{13}C-NMR measurements of mexazolam and the 3-methyl compound were carried out at 25 °C in aqueous buffer containing 4% (v/v) ethanol with $\mu = 0.1$ M (NaCl).

Results and Discussion

pH–Rate Profile for Acid–Base Equilibrium of Benzodiazepinooxazoles

Figure 1 illustrates the UV spectra of cloxazolam in various pH buffer solutions. These spectra are attributable to equilibrium mixtures of the ring-opened iminium form (AF) in acid solution and the ring-closed form (BF) in weakly alkaline solution (see Chart 2 for structures).[2-4] From the spectral data, similarly to the case of oxazolam studied previously,[2] the apparent pK_{eq} value ($-\log([BF][H^+]/[AF])$) and the $pK_{a,2}$ value ($-\log([BA][H^+]/[BF])$) were estimated as 6.90 and 10.8, respectively. These values agree fairly well with those in the literature.[7-9]

Figure 2 shows the pH–rate profiles for the acid–base equilibria of cloxazolam and flutazolam. The shape of the profile for cloxazolam is similar to that for oxazolam reported previously,[2] suggesting that the reaction scheme shown in Chart 2 is applicable. In Chart 2 $K'_{a,2}$ and $K_{a,2}$ are the dissociation constants of AF and BF, respectively, these processes being much faster than the processes of ring-opening and -closing.[2] The presence of the processes corresponding to $K'_{a,2}$ and $K_{a,2}$ is supported by the absence of the inflection at the weakly alkaline region in the pH–rate profile of flutazolam and by the absence of the spectral change with the strongly alkaline flutazolam solutions, respectively. Flutazolam carries a 2-

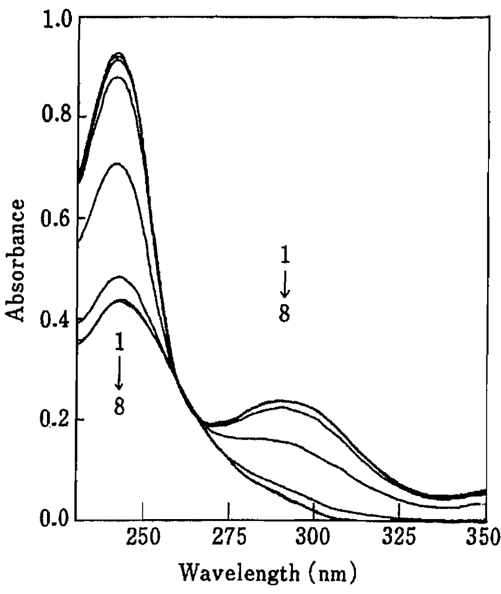

Fig. 1. UV Absorption Spectra of Cloxazolam at Various pH Values

Concentration of cloxazolam, 3.00×10^{-5} M.
1, pH 3.03; 2, 3.94; 3, 5.01; 4, 6.00; 5, 7.00; 6, 8.02; 7, 9.00; 8, 9.99.

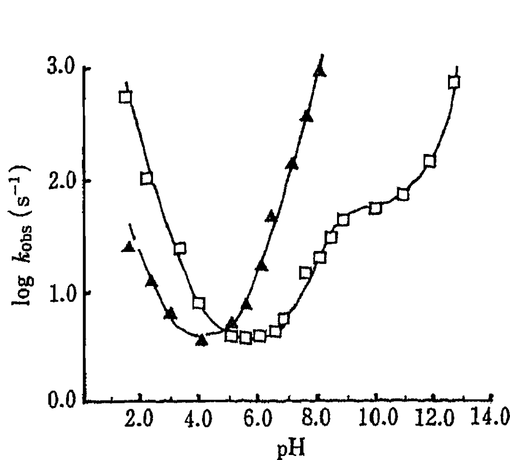

Fig. 2. The pH-Rate Profiles for Oxazolidine Ring-Opening and Ring-Closing Reactions of Cloxazolam and Flutazolam at 25 °C

□, cloxazolam; ▲, flutazolam.

AF ⇌ BF + H^+ ($k^0_{Cl,F}$, $k^{OH^-}_{Cl,F}$ [OH^-]; $k^0_{Op,F}$ [H^+], $k^{H^+}_{Op,F}$ [H^+])

AF ⇌ AA + H^+ ($K'_{a,2}$); BF ⇌ BA + H^+ ($K_{a,2}$)

AA ⇌ BA + H^+ ($k^0_{Cl,A}$, $k^{OH^-}_{Cl,A}$ [OH^-]; $k^0_{Op,A}$, $k^{H^+}_{Op,A}$ [H^+])

Chart 2

hydroxyethyl group instead of the dissociative hydrogen atom at position 7. In Chart 2 the rate constants represent the following reactions: $k^0_{Cl,F}$, water-catalyzed ring-closing from the free form (AF) of nitrogen at position 7; $k^{OH^-}_{Cl,F}$, hydroxide ion-catalyzed ring-closing; $k^{H^+}_{Op,F}$, hydrogen ion-catalyzed ring-opening from the free form (BF) of the nitrogen; $k^0_{Op,F}$, water-

TABLE I. Estimated Rate Constants and Equilibrium and Dissociation Constants for Cloxazolam, Haloxazolam, and Flutazolam[a)]

	$k_{Op,F}^{H^+}$ $s^{-1}M^{-1}$	$k_{Op,F}^{0}+k_{Cl,F}^{0}$ s^{-1}	$k_{Cl,F}^{OH^-}$ $s^{-1}M^{-1}$	$k_{Cl,A}^{0}$ s^{-1}	$k_{Cl,A}^{OH^-}$ $s^{-1}M^{-1}$	$K'_{a,2}$ M^{-1} ($pK'_{a,2}$)	$K_{a,2}$ M^{-1} ($pK_{a,2}$)	K_{eq} M^{-1} (pK_{eq})
Cloxazolam	2.72×10^{4}	3.97	2.27×10^{7}	1.05×10^{-1}	1.62×10^{4}	3.61×10^{-9} (8.44)	1.58×10^{-11} (10.8)	1.26×10^{-7} (6.90)
Haloxazolam	2.55×10^{4}	2.06	1.55×10^{8}	8.25×10^{-1}	7.07×10^{4}	4.02×10^{-9} (8.94)	2.00×10^{-12} (11.7)	7.94×10^{-7} (6.10)
Flutazolam	2.03×10^{3}	3.92	1.21×10^{9}	—	—	—	—	2.51×10^{-6} (5.60)

a) 25 °C; containing 4% (v/v) ethanol.

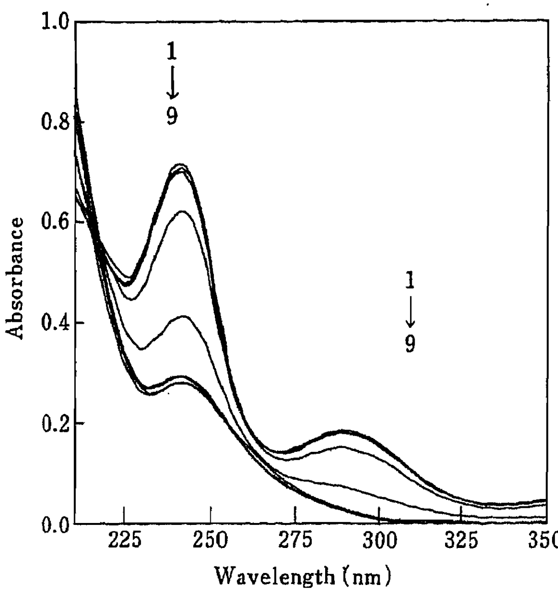

Fig. 3. UV Absorption Spectra of Mexazolam at Various pH Values

Concentration of mexazolam, 3.00×10^{-5} M.
1, pH 3.17[a)]; 2, 4.04[a)]; 3, 5.06[a)]; 4, 6.00; 5, 7.00; 6, 8.02; 7, 9.00; 8, 9.99; 9, 10.99.
a) The concentrations of the buffer constituents were one third or one sixth of the ordinary buffer concentrations used for the kinetic experiments.

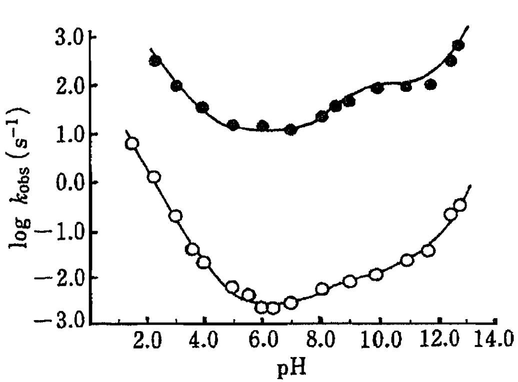

Fig. 4. The pH–Rate Profiles for Oxazolidine Ring-Opening and Ring-Closing Reactions of Mexazolam at 25 °C

●, fast step reaction; ○, slow step reaction.

catalyzed ring-opening; $k_{Cl,A}^{0}$, water-catalyzed ring-closing from the anionic form (AA) of the nitrogen; $k_{Cl,A}^{OH^-}$, hydroxide ion-catalyzed ring-closing; $k_{Op,A}^{H^+}$, hydrogen ion-catalyzed ring-opening from the anionic form (BA) of the nitrogen; $k_{Op,A}^{0}$, water-catalyzed ring-opening.

The rate constants and dissociation constants in Chart 2 were determined by methods similar to those used in the case of oxazolam reported previously.[2)] The obtained values, together with the pK_{eq} value, are summarized in Table I. The solid curves in Fig. 2 were calculated by applying the values in Table I to equations which were derived in the way similar to that used for oxazolam.[2)] The differences in the hydroxide ion-catalyzed ring-closing rate constants ($k_{Cl,F}^{OH^-}$ and $k_{Cl,A}^{OH^-}$) between cloxazolam and haloxazolam may reflect the difference in the steric effect, rather than in the electronegativity, between the chlorine atom and fluorine atom at position 2′.[10)]

Kinetics and Mechanism of Acid–Base Equilibrium of Mexazolam

The UV spectral changes with pH for mexazolam are shown in Fig. 3. The analysis of the spectra, done in the same way as for other benzodiazepinooxazoles, gave the pK_{eq} value of 6.90, in fair agreement with the literature value of 6.60.[7]

Figure 4 shows the pH–rate profile for the acid–base equilibrium of mexazolam. Unlike other benzodiazepinooxazoles, two step reactions (fast step ($k_{obs,F}$, ●) and slow step ($k_{obs,S}$, ○)) are found over the whole pH range. The ratio of $k_{obs,F}$ to $k_{obs,S}$ ranges from 10^3 to 10^4. For the ring-opening reaction (initiated by pH-jump, for example, from pH 9.0 to 4.0), the ratio of the absorbance increment at 240 nm due to the fast step ($k_{obs,F}$) to that due to the slow step ($k_{obs,S}$) was about 1 : 6. For the ring-closing reaction (initiated by pH-jump, for example, from pH 3.0 to 9.0), on the other hand, the ratio of the absorbance decrement based on $k_{obs,F}$ to that based on $k_{obs,S}$ was about 4 : 1. From these results of the rate ratio and the absorbance ratio, we propose the reaction mechanism shown in Chart 3 in order to explain the pH–rate profiles for mexazolam. The reaction scheme involves an intermediate (IF) detected by the kinetic method. The absorbance changes due to the reaction process of AF⇄IF are larger than those due to IF⇄BF. The interrelation of the rates, *i.e.*, fast step or slow step, is denoted in Chart 3.

As has been reported previously,[2] oxazolam showed two pH–rate profiles in the acid region due to the *cis* and *trans* isomers (BF_{cis} and BF_{trans}, referring to the 2-methyl group and 11b-phenyl group). The profiles were analyzed according to the reaction scheme shown in Chart 4.[2] For the following reasons, we chose the reaction scheme shown in Chart 3 rather than that in Chart 4 for mexazolam: (1) the ^{1}H-NMR spectrum of mexazolam in methanol-d_4 indicates the existence of essentially only one species, and the ^{13}C-NMR spectrum in chloroform-*d* shows a single signal at δ 97.5 assigned to the carbon atom at position 11b[11]; (2) by using the reaction scheme shown in Chart 4 the ratio of $k_{obs,F}$ to $k_{obs,S}$ can not be related reasonably with the ratio of the absorbance change from the fast step to that from the slow step; (3) two step reactions are observed over the whole pH range for the acid–base equilibrium of mexazolam.

In order to determine whether the 2′-chlorine atom or the 3-methyl group of mexazolam causes the difference in the reaction mechanism between mexazolam (Chart 3) and oxazolam (Chart 4), the 3-methyl compound (see Chart 1 for the chemical structure) was synthesized

AF ⇄ (fast step →, ← slow step) [N / OH / HO / CH_3 and/or N---H^+ / O / CH_3 IF] ⇄ (slow step →, ← fast step) $BF + H^+$

Chart 3

$$H^+ + BF_{cis} \rightleftharpoons AF \rightleftharpoons BF_{trans} + H^+$$

Chart 4

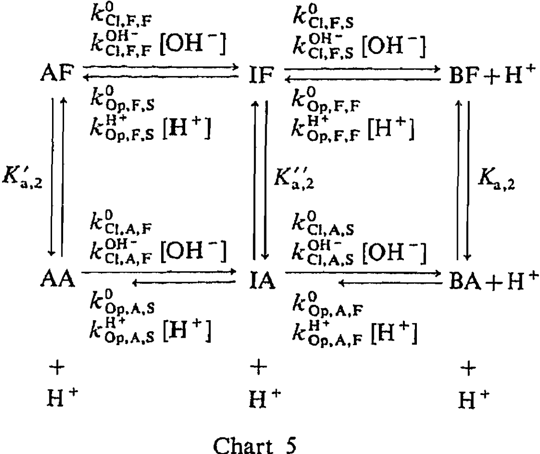

Chart 5

TABLE II. Estimated Rate Constants and Dissociation Constants for Mexazolam and the 3-Methyl Compounds[a)]

	$k^{H^+}_{Op,F,F}$ $s^{-1}M^{-1}$	$k^0_{Op,F,F}+k^0_{Cl,F,F}$ s^{-1}	$k^{OH^-}_{Cl,F,F}$ $s^{-1}M^{-1}$	$k^0_{Cl,A,F}$ s^{-1}	$k^{OH^-}_{Cl,A,F}$ $s^{-1}M^{-1}$	$K'_{a,2}$ M^{-1} ($pK'_{a,2}$)	$K_{a,2}$ M^{-1} ($pK_{a,2}$)	K_{eq} M^{-1} (pK_{eq})
Mexazolam	5.36×10^5	1.15×10	1.20×10^7	1.69	1.43×10^4	8.55×10^{-10} (9.07)	6.31×10^{-13} (12.2)	1.26×10^{-7} (6.9)
3-Methyl compound	8.12×10^4	2.35	1.30×10^8	Negligible	4.40×10^3	1.10×10^{-8} (7.96)	1.00×10^{-12} (12.0)	6.31×10^{-7} (6.2)

	$k^{H^+}_{Op,F,S}$ $s^{-1}M^{-1}$	$k^0_{Op,F,S}+k^0_{Cl,F,S}$ s^{-1}	$k^{OH^-}_{Cl,F,S}$ $s^{-1}M^{-1}$	$k^0_{Cl,A,S}$ s^{-1}	$k^{OH^-}_{Cl,A,S}$ $s^{-1}M^{-1}$	$K''_{a,2}$ M^{-1} ($pK''_{a,2}$)
Mexazolam	1.93×10^2	2.87×10^{-3}	2.97×10^3	1.98×10^{-3}	7.43	1.94×10^{-9} (8.72)
3-Methyl compound	1.82×10^2	1.76×10^{-3}	5.09×10^4	Negligible	1.00×10	5.03×10^{-8} (7.30)

a) 25 °C; containing 4% (v/v) ethanol.

and kinetic studies were carried out. The pH–rate profiles for the 3-methyl compound were very similar to those for mexazolam, indicating the cause of the difference to be the 3-methyl group rather than the 2′-chlorine atom. The pH–rate profile for cloxazolam, which contains a 2′-chlorine atom and lacks the 3-methyl group (Fig. 2) (one step reaction), also supports the hypothesis that the 3-methyl group of mexazolam is the cause of the difference in the mechanism.

The reaction scheme shown in Chart 5 can account for the pH–rate profiles of mexazolam illustrated in Fig. 4. The superscripts and subscripts of the rate constants in Chart 5 have the following meanings. The superscripts 0, H^+, and OH^- represent the water-catalyzed, hydrogen ion-catalyzed, and hydroxide ion-catalyzed reactions, respectively. The first subscript indicates whether ring-opening (Op) or ring-closing (Cl) occurs, the second (intermediate) one indicates the free form (F) at the 7 nitrogen atom of mexazolam or the anionic form (A), and the last one indicates the fast reaction (F) or the slow reaction (S). $K''_{a,2}$ is the dissociation constant of IF.

According to Chart 5, the apparent first order rate constants for the fast reaction $k_{obs,F}$ and for the slow reaction $k_{obs,S}$ are given by Eqs. 1 and 2, respectively.

$$k_{obs,F}=k^{H^+}_{Op,F,F}[H^+]+k^{0}_{Op,F,F}+\{1/([H^+]+K'_{a,2})\}$$
$$\times\{k^{0}_{Cl,F,F}[H^+]+k^{OH^-}_{Cl,F,F}K_W+k^{0}_{Cl,A,F}K'_{a,2}$$
$$+k^{OH^-}_{Cl,A,F}(K_W/[H^+])K'_{a,2}\} \qquad (1)$$

$$k_{obs,S}=k^{H^+}_{Op,F,S}[H^+]+k^{0}_{Op,F,S}+\{1/([H^+]+K''_{a,2})\}$$
$$\times\{k^{0}_{Cl,F,S}[H^+]+k^{OH^-}_{Cl,F,S}K_W+k^{0}_{Cl,A,S}K''_{a,2}$$
$$+k^{OH^-}_{Cl,A,S}(K_W/[H^+])K''_{a,2}\} \qquad (2)$$

By procedures similar to those employed in the case of oxazolam, each parameter was estimated and is listed in Table II. The solid curves in Fig. 4 were simulated by using these parameters. Comparing the results for mexazolam and the 3-methyl compound, it is clear that the 2′-chlorine atom greatly affects the hydroxide ion-catalyzed ring-closing reactions ($k^{OH^-}_{Cl,F,F}$ and $k^{OH^-}_{Cl,F,S}$).

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Studies on Poisonous Metals. XVIII.[1] Effects of Several Dithiocarbamates on Tissue Distribution and Excretion of Cadmium in Rats

SHOJI KOJIMA,* MORIO KIYOZUMI, TOSHIYA HONDA, YOSHINOBU SENBA, KAZUYOSHI KAMINAKA, and MIZUO OHNISHI

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862, Japan

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The effects of various chelating agents, sodium *N*-benzyl-D-glucamine dithiocarbamate (NBGDTC), sodium *N*-*p*-methylbenzyl-D-glucamine dithiocarbamate (NMBGDTC), and sodium *N*-*p*-isopropylbenzyl-D-glucamine dithiocarbamate (NPBGDTC), which were newly synthesized, sodium *N*-methyl-D-glucamine dithiocarbamate (NMGDTC), diethyldithiocarbamate (DEDTC), dihydroxyethyldithiocarbamate (DHEDTC), and dicarboxymethyldithiocarbamate (DCMDTC), on the distribution and excretion of cadmium in rats were compared at 30 min and 24 h following cadmium injection. At 30 min after cadmium administration, these chelating agents all significantly enhanced the biliary excretion of cadmium, and at 24 h after cadmium injection, the chelating agents other than DEDTC and DCMDTC significantly enhanced the biliary excretion of the metal. The effects of NBGDTC, NMBGDTC, and NPBGDTC on the biliary excretion of cadmium were much larger than those of the other chelating agents. The urinary excretion of cadmium was increased by the chelating agents other than DEDTC and NPBGDTC. These chelating agents were effective in mobilizing cadmium from the liver, kidney, and pancreas at 30 min after treatment with cadmium. At both 30 min and 24 h after treatment with cadmium, NBGDTC, NMBGDTC, and NPBGDTC effectively depressed cadmium content in the liver without the mobilization of cadmium to the kidney. The chelating agents other than DEDTC did not result in the redistribution of cadmium to the brain, testes, lung, and heart. These results show that the injection of NBGDTC, NMBGDTC, or NPBGDTC at both 30 min and 24 h after treatment with cadmium can much more effectively mobilize cadmium from the body, mainly through the bile, without redistribution of cadmium to other tissues, such as brain, testes, lung, and heart, when compared with injection of other chelating agents. Furthermore, the pattern of mobilization and excretion of cadmium following treatment with each chelating agent was related to the organic/aqueous partition coefficient of each dithiocarbamate–cadmium complex.

Keywords——cadmium; tissue distribution; excretion; dithiocarbamate; chelate effect; partition coefficient

A single injection of cadmium into animals may result in a number of lesions in various organs, such as liver, testes, and sensory ganglia.[2] However, the kidney is the most sensitive organ to long-term exposure to cadmium, *i.e.*, the critical organ for this type of exposure.[3,4] Therefore, it is important to develop an effective chelator therapy for removal of cadmium to prevent cadmium-induced diseases. It has been reported that the biliary excretion of cadmium is an important excretory route after exposure to cadmium.[5-8] Since Sunderman achieved success in treating experimental and clinical nickel poisoning with diethyldithiocarbamate (DEDTC),[9-12] various dithiocarbamate compounds have been used in experimental cadmium poisoning in animals. DEDTC was more effective when treatment was delayed for some time than when it was given prior to or immediately after cadmium administration.[13,14] Some recent studies showed that injections of dithiocarbamates, such as dimethyldithiocarbamate,[15] DEDTC,[15-18] and diisopropyldithiocarbamate,[15] to mice pretreated with $CdCl_2$ 2 weeks

earlier, extensively reduced hepatic and renal cadmium burdens, but increased the cadmium load in the brain, testes, and heart. Repeated intraperitoneal injection of ammonium dihydroxydithiocarbamate (DHEDTC)[17] and sodium *N*-methyl-D-glucamine dithiocarbamate (NMGDTC)[19,20] into mice following exposure to cadmium resulted in substantial reduction in both the liver and kidney concentrations of cadmium without redistribution of cadmium to brain, testes, and heart. In addition, we reported that the injection of sodium *N*-benzyl-D-glucamine dithiocarbamate (NBGDTC), which was newly synthesized by us, into rats treated with cadmium was more effective in decreasing the cadmium concentration in the liver and kidney without the mobilization of cadmium to the kidney and without redistribution of cadmium to tissues such as the brain, testes, and heart, than injection of 2,3-dimercaptopropanol (BAL) or NMGDTC.[21,22]

In order to develop better chelating agents to mobilize cadmium from the body, we studied the comparative effects of NBGDTC, sodium *N*-*p*-methylbenzyl-D-glucamine dithiocarbamate (NMBGDTC), and sodium *N*-*p*-isopropylbenzyl-D-glucamine dithiocarbamate (NPBGDTC), which were newly synthesized by us, as well as NMGDTC, DEDTC, DHEDTC, and ammonium dicarboxymethyldithiocarbamate (DCMDTC), on the biliary and urinary excretion and tissue distribution of cadmium in rats at 30 min and 24 h after pretreatment with cadmium.

Experimental

Materials——^{109}Cd (specific activity, 1574 mCi/mg) was obtained from New England Nuclear (Boston, Mass.). Cadmium chloride and DEDTC were obtained from Wako Pure Chemical Ind. (Osaka). NMGDTC was prepared according to the procedure of Shinobu *et al.*[19] DHEDTC and DCMDTC were prepared by the reported methods.[17] NBGDTC was synthesized according to the procedure reported in our previous paper.[21] NMBGDTC and NPBGDTC were synthesized by the use of *N*-*p*-methylbenzyl-D-glucamine and *N*-*p*-isopropylbenzyl-D-glucamine,

$NaS-C(=S)-N(CH_2CH_3)_2$

sodium diethyldithiocarbamate (DEDTC)

$NH_4S-C(=S)-N(CH_2CH_2OH)_2$

ammonium dihydroxyethyldithiocarbamate (DHEDTC)

$NH_4S-C(=S)-N(CH_2COONH_4)_2$

ammonium dicarboxymethyldithiocarbamate (DCMDTC)

$NaS-C(=S)-N(CH_3)CH_2(CHOH)_4CH_2OH$

sodium *N*-methyl-D-glucamine dithiocarbamate (NMGDTC)

$NaS-C(=S)-N(CH_2C_6H_5)CH_2(CHOH)_4CH_2OH$

sodium *N*-benzyl-D-glucamine dithiocarbamate (NBGDTC)

$NaS-C(=S)-N(CH_2C_6H_4CH_3)CH_2(CHOH)_4CH_2OH$

sodium *N*-*p*-methylbenzyl-D-glucamine dithiocarbamate (NMBGDTC)

$NaS-C(=S)-N(CH_2C_6H_4CH(CH_3)_2)CH_2(CHOH)_4CH_2OH$

sodium *N*-*p*-isopropylbenzyl-D-glucamine dithiocarbamate (NPBGDTC)

Fig. 1. Structures of Dithiocarbamates Used

respectively, according to the method of our previous paper.[21] The products were analyzed, and the results were as follows. NMBGDTC. *Anal.* Calcd for $C_{15}H_{22}NNaO_5S_2 \cdot H_2O$: C, 44.87; H, 5.53; N, 3.49. Found: C, 44.35; H, 5.33; N, 3.60. NPBGDTC. *Anal.* Calcd for $C_{17}H_{26}NNaO_5S_2$: C, 49.61; H, 6.38; N, 3.40. Found: C, 49.12; H, 6.28; N, 3.31. Structural formulas of dithiocarbamates used are shown in Fig. 1. All other chemicals were of reagent grade.

Distribution and Excretion Studies——Male Wistar rats, weighing 190—230 g, were injected intraperitoneally with $^{109}CdCl_2$ (1 mg of Cd and 10 μCi of ^{109}Cd/kg) in 0.5 ml of physiological saline and housed in individual metabolic cages with drinking water and diet (Nosan Lab Chow) *ad libitum*. At 30 min or 24 h later, the rats were anesthetized with urethane (1 g/kg intraperitoneally) and the bile duct was cannulated with polyethylene tubing (PE 10) as described previously.[8] The rats were given intraperitoneally saline (control) or a chelating agent (400 μmol/kg) in 0.5 ml of saline. Bile and urine samples were collected for an experimental period of 3 h. Then, the rats were killed with urethane and various tissues were collected. The levels of ^{109}Cd radioactivity in the bile, urine and tissues were determined using an Aloka auto-well gamma scintillation counter (model ARC 300).

Determination of Partition Coefficients——The *n*-octanol/aqueous partition coefficient of each chelating agent–cadmium complex was determined according to the procedure in our previous paper[21] using 0.1 M Tris buffer (pH 7.4) as the aqueous solvent. The partition coefficients were expressed as $\log_{10}$ (cpm of ^{109}Cd in the *n*-octanol phase/cpm of ^{109}Cd in the aqueous phase).

Statistical Analysis——Data were compared by analysis of variance. When the analysis indicated a significant difference, the treated groups were compared to controls by means of Student's *t* test.

Results and Discussion

Table I shows the biliary and urinary excretions of cadmium when each chelating agent was injected 30 min after cadmium challenge. The results show that the major route of excretion of cadmium after injection of these chelating agents other than DCMDTC into cadmium-exposed rats was *via* the bile. The biliary excretion of cadmium was significantly increased by injection of these chelating agents and was remarkedly enhanced by treatment with DHEDTC, NMGDTC, NBGDTC, NMBGDTC, or NPBGDTC. In particular, benzyl- or alkylbenzyl-D-glucamine dithiocarbamates (NBGDTC, NMBGDTC, and NPBGDTC) showed an excellent enhancing effect on the biliary excretion of cadmium. In addition, DHEDTC, NMGDTC, and NBGDTC increased the urinary excretion of cadmium to a small extent, although DEDTC and NPBGDTC decreased the urinary excretion

TABLE I. Effects of Chelating Agents on Biliary and Urinary Excretions of Cadmium in Rats Pretreated with Cadmium, 30 min and 24 h Earlier

Chelating agent	Cadmium excreted/3 h (% of dose)[a]			
	Cadmium pretreatment			
	30 min earlier		24 h earlier	
	Bile	Urine	Bile	Urine
Control	8.23±0.61	0.005±0.001	0.003±0.002	0.005±0.004
DEDTC	0.54±0.11[d]	0.002±0.001[b]	0.004±0.002	0.031±0.018
DHEDTC	24.60±3.28[b]	0.23 ±0.01[d]	0.21 ±0.08[b]	0.034±0.010[d]
DCMDTC	2.52±2.00[d]	3.96 ±1.35[e]	0.003±0.001	0.10 ±0.05
NMGDTC	28.02±0.58[e]	1.25 ±0.12[d]	0.33 ±0.11[b]	0.23 ±0.05[d]
NBGDTC	46.36±2.20[e]	0.34 ±0.05[c]	10.32 ±2.44[d]	0.23 ±0.12[b]
NMBGDTC	42.91±0.95[e]	0.082±0.029	21.47 ±2.38[e]	0.085±0.040[b]
NPBGDTC	38.17±2.48[e]	0.001±0.001[c]	19.02 ±2.00[e]	0.002±0.001

The rats were injected with $^{109}CdCl_2$ (1 mg of Cd and 10 μCi of ^{109}Cd/kg). At 30 min or 24 h later they were injected intraperitoneally with saline or chelating agents (400 μmol/kg) and then bile and urine samples were collected for 3 h. *a*) The values represent the mean ± S.D. for 3 to 5 animals. Significantly different from the control values: *b*) $p<0.05$; *c*) $p<0.02$; *d*) $p<0.01$; *e*) $p<0.001$.

TABLE II. Effects of Chelating Agents on Tissue Distribution of Cadmium in Rats Pretreated with Cadmium 30 min Earlier

Chelating agent	Cadmium (% of dose)[a]							
	Liver	Kidney	Pancreas	Heart	Testes	Lung	Spleen	Brain
Control	59.03±3.54	2.05±0.15	1.54±0.09	0.30±0.04	0.17±0.02	0.23±0.04	0.13 ±0.02	0.031±0.002
DEDTC	55.62±2.15	1.64±0.15[c]	0.96±0.12[d]	0.29±0.02	0.13±0.01[b]	0.55±0.09[d]	0.22 ±0.04[c]	0.26 ±0.02[e]
DHEDTC	41.07±3.51[d]	2.28±0.29	1.03±0.23[b]	0.21±0.03	0.15±0.004	0.16±0.04	0.085±0.021	0.024±0.005
DCMDTC	52.72±2.44[b]	2.05±0.16	1.10±0.11[d]	0.26±0.03	0.16±0.01	0.19±0.01	0.082±0.010[c]	0.029±0.002
NMGDTC	27.21±1.19[d]	1.55±0.15	1.08±0.06[d]	0.24±0.03	0.17±0.02	0.14±0.02[b]	0.11 ±0.001	0.032±0.005
NBGDTC	21.14±3.42[e]	1.29±0.12[d]	1.08±0.11[d]	0.22±0.02	0.14±0.01	0.14±0.01	0.10 ±0.01	0.024±0.004
NMBGDTC	21.07±1.17[e]	1.37±0.01[b]	0.95±0.13[d]	0.21±0.01[b]	0.14±0.01	0.15±0.01[b]	0.089±0.008[b]	0.027±0.002
NPBGDTC	18.65±1.51[d]	1.41±0.13[c]	1.07±0.16[b]	0.23±0.01	0.18±0.01	0.21±0.02	0.15 ±0.04	0.022±0.002

The rats were injected intraperitoneally with $^{109}CdCl_2$ (1 mg of Cd and 10 μCi of ^{109}Cd/kg). After 30 min, they were injected intraperitoneally with saline or chelating agents (400 μmol/kg). At the end of the experiment, the rats were killed and the tissue distribution of cadmium was determined from the radioactivity. *a*) Each value represents the mean±S.D. for 3 to 5 animals. Significantly different from the control values: *b*) $p<0.05$; *c*) $p<0.02$; *d*) $p<0.01$; *e*) $p<0.001$.

TABLE III. Effects of Chelating Agents on Tissue Distribution of Cadmium in Rats Pretreated with Cadmium 24 h Earlier

Chelating agent	Cadmium (% of dose)[a]							
	Liver	Kidney	Pancreas	Heart	Testes	Lung	Spleen	Brain
Control	52.99±4.12	2.07±0.40	3.09±1.41	0.18±0.04	0.20±0.05	0.24±0.05	0.21±0.07	0.039±0.008
DEDTC	52.14±5.95	2.42±0.24	1.71±0.79	0.19±0.01	0.19±0.02	0.25±0.04	0.21±0.05	0.053±0.004[b]
DHEDTC	57.08±7.73	2.18±0.03	1.18±0.02	0.19±0.04	0.21±0.06	0.21±0.03	0.32±0.06	0.022±0.003[d]
DCMDTC	59.06±6.43	2.15±0.05	1.35±0.28	0.15±0.004	0.18±0.01	0.19±0.02	0.23±0.06	0.024±0.001
NMGDTC	56.29±4.23	2.46±0.29	1.59±0.56	0.17±0.03	0.19±0.04	0.17±0.02	0.24±0.04	0.040±0.008
NBGDTC	42.70±2.16[b]	1.93±0.17	1.73±0.69	0.19±0.03	0.15±0.01	0.15±0.01	0.22±0.04	0.027±0.004
NMBGDTC	28.69±1.72[e]	2.04±0.09	2.03±0.13	0.20±0.03	0.19±0.04	0.15±0.03[b]	0.20±0.05	0.030±0.006
NPBGDTC	39.72±4.48[c]	2.03±0.10	1.66±0.71	0.17±0.01	0.20±0.02	0.19±0.01	0.31±0.04	0.027±0.005[b]

The rats were injected intraperitoneally with $^{109}CdCl_2$ (1 mg of Cd and 10 μCi of ^{109}Cd/kg). After 24 h, they were injected intraperitoneally with saline or chelating agents (400 μmol/kg). At the end of the experiment, the rats were killed and the tissue distribution of cadmium was determined from the radioactivity. *a*) Each value represents the mean±S.D. for 3 to 5 animals. Significantly different from the control values: *b*) $p<0.05$; *c*) $p<0.02$; *d*) $p<0.01$; *e*) $p<0.001$.

of cadmium. DCMDTC among the chelating agents used showed the largest stimulatory effect on the urinary excretion of cadmium, and the major route of excretion of cadmium after injection of this chelating agent in cadmium-exposed rats was *via* the urine.

The tissue distribution of cadmium in rats treated with $^{109}CdCl_2$ and each chelating agent at the end of the experiment is shown in Table II. The percentages of cadmium deposited in the liver, kidney, and pancreas were significantly decreased by most of these chelating agents. The D-glucamine dithiocarbamate analogs (NMGDTC, NBGDTC, NMBGDTC, and NPBGDTC) remarkably reduced the cadmium content in the liver. DEDTC caused undesirable redistribution of cadmium to the tissues, such as brain, lung, and spleen. Gale *et al.*[15,16] reported that injections of dithiocarbamates, such as DEDTC and dimethyldithiocarbamate, into mice pretreated with cadmium caused the redistribution of cadmium to the brain, testes, and heart. However, NBGDTC, NMBGDTC, and NPBGDTC, which were synthesized by us, did not cause the redistribution of cadmium to brain, lung, testes, and heart.

The induction of metallothionein synthesis within a few hours after cadmium exposure has a marked effect on the pharmacokinetics and toxicity of cadmium.[23] Thus, we examined the comparative effects of these chelating agents on the biliary and urinary excretions of cadmium when administered 24 h after pretreatment with cadmium, *i.e.*, after the synthesis of metallothionein (Table I). The excretion of cadmium in the bile, which is the major excretory

TABLE IV. Partition Coefficient of Cadmium Ion and Cadmium Complexes with Chelating Agents in *n*-Octanol/Aqueous System

Reaction mixture	Partition coefficient[a] 0.1 M Tris buffer	Reaction mixture	Partition coefficient[a] 0.1 M Tris buffer
Cd ion only	−3.13±0.11	Cd+NMGDTC	−3.08±0.02
Cd+DEDTC	3.77±0.04	Cd+NBGDTC	0.30±0.01
Cd+DHEDTC	−0.86±0.01	Cd+NMBGDTC	1.35±0.02
Cd+DCMDTC	−4.75±0.17	Cd+NPBGDTC	3.16±0.21

a) Expressed as $\log_{10}$(cpm in *n*-octanol phase/cpm in aqueous phase) at 37 °C. Each value represents the mean±S.D. for 3 individual measurements.

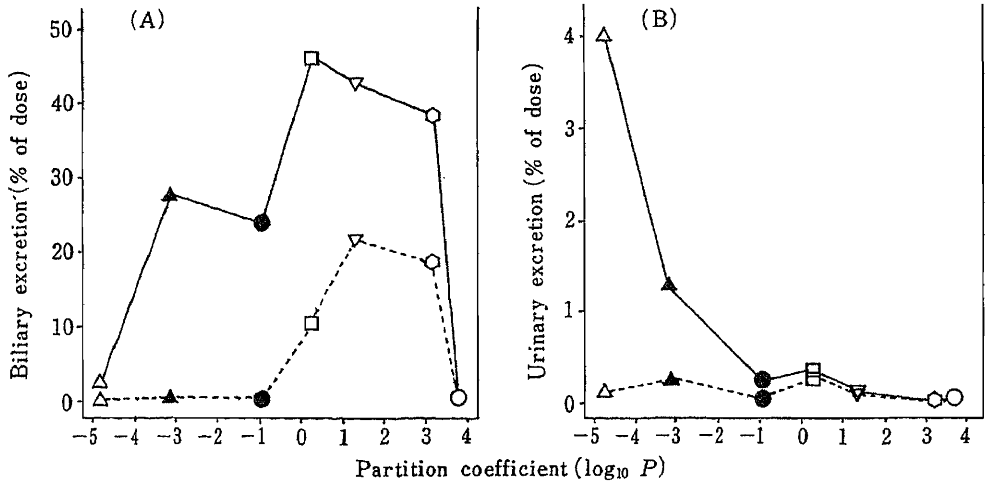

Fig. 2. Relationship between Biliary (A) or Urinary Excretion (B) of Cadmium and Partition Coefficient of Cadmium-Chelating Agent Complex

○, DEDTC; ●, DHEDTC; △, DCMDTC; ▲, NMGDTC; □, NBGDTC; ▽, NMBGDTC; ⬡, NPBGDTC. ——, 30 min; -----, 24 h.

route of cadmium, was significantly increased by injection of DHEDTC, NMGDTC, NBGDTC, NMBGDTC, or NPBGDTC; NBGDTC, NMBGDTC, and NPBGDTC were the most effective. However, the delayed treatment with these chelating agents was less effective on the biliary excretion of cadmium than that at 30 min after cadmium exposure. The urinary excretion of cadmium was increased to a small extent by treatment with DHEDTC, NMGDTC, NBGDTC, and NMBGDTC, but was little affected by treatment with the other chelating agents.

Table III shows the tissue distribution of cadmium at 3 h after injection of the chelating agents into rats pretreated with cadmium 24 h earlier. Only NBGDTC, NMBGDTC, and NPBGDTC among these chelating agents significantly reduced the percentage of cadmium in the liver, the major site of accumulation of cadmium in the body, without the mobilization of cadmium to the kidney, and also tended to decrease the cadmium contents in the pancreas, lung, and brain. These results indicate that the injections of these 3 chelating agents can mobilize metallothionein-bound cadmium from the rat body even after the synthesis of metallothionein, leading to excretion of the metal mainly through the bile, as reported in our previous paper.[1,21] In addition, in the pretreatment with the chelating agents other than DEDTC, the undesirable redistribution of cadmium to the brain, testes, lung, and heart was not observed.

The partition coefficients of the complexes of cadmium with the chelating agents are listed in Table IV. In contrast to DEDTC, NPBGDTC, and NMBGDTC, which yield lipid-soluble complexes with cadmium, the complexes of cadmium with DCMDTC and NMGDTC had greater lipophobicity, and the complexes of cadmium with NBGDTC and DHEDTC yielded slightly positive and negative partition coefficient ($\log_{10}P$) values, respectively, indicating intermediate lipophobicity. Figure 2 shows the relationship between the biliary or urinary excretion of cadmium and the $\log_{10}P$ of the cadmium–chelating agent complex. In the treatment with the chelating agents at 30 min after cadmium exposure, the dithiocarbamates such as NMGDTC, DHEDTC, NBGDTC, NMBGDTC, and NPBGDTC, which form complexes with cadmium having $\log_{10}P$ values of about -3 to 3, remarkably enhanced the biliary excretion of cadmium, resulting in decreased cadmium contents in the liver, kidney, and pancreas. On the other hand, in the treatment with the chelating agents at 24 h after cadmium exposure, NBGDTC, NMBGDTC, and NPBGDTC, which form complexes having $\log_{10}P$ values of about 0 to 3, showed much larger stimulatory effects on the biliary excretion of cadmium, resulting in the decrease of cadmium content in the liver. In addition, NMGDTC and DCMDTC, which from complexes having $\log_{10}P$ values of about -3 to -5, appeared to increase the urinary excretion of cadmium. DEDTC, which forms a complex having a $\log_{10}P$ value of about 4, hardly increased the biliary or urinary excretion of cadmium, and caused the redistribution of cadmium to the brain. Jones and Jones[24] have suggested that changes in the relative hydrophobic/hydrophilic character of the groups attached to the nitrogen atom of dithiocarbamates may be important in determining the antidotal activity of the compounds. The results of the present study also suggest that the pattern of distribution and excretion of cadmium after treatment with these chelating agents is related to the partition coefficients of the the cadmium–dithiocarbamate complexes and that NBGDTC, NMBGDTC, and NPBGDTC, in which the complexes with cadmium exhibit $\log_{10}P$ values of about 0 to 3, are the most effective dithiocarbamates among the compouds examined in removing cadmium from the body (mainly about the bile) without the mobilization of cadmium to the kidney and without the redistribution of cadmium to other tissues, such as brain, testes, lung, and heart, at both 30 min and 24 h after treatment with cadmium.

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Notes

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Enzymes and Catalysts. II.[1] Pig Liver Esterase-Catalyzed Asymmetric Synthesis of (−)- and (+)-Cucurbitine and Its (−)-Thio Analogue

YOSHIHISA MORIMOTO and KAZUO ACHIWA*

Shizuoka College of Pharmacy, 2–2–1 Oshika, Shizuoka 422, Japan

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The asymmetric synthesis of (−)- and (+)-cucurbitine was carried out by a method involving 1,3-dipolar cycloaddition of the intermediary azomethine ylide and pig liver esterase (PLE)-catalyzed hydrolysis. The thio analogue, (−)-3-aminotetrahydrothiophene-3-carboxylic acid, was also synthesized.

Keywords——cucurbitine; pig liver esterase; 1,3-dipolar cycloaddition; pyrrolidine; tetrahydrothiophene; half ester; asymmetric synthesis

(−)-Cucurbitine ((−)-**1**),[2] an unusual amino acid isolated from the seeds of several species of *Cucurbitaceae*, is known to inhibit the growth of immature *Schistosoma japonicum*. Asymmetric synthesis of this structurally interesting compound has not been reported.[3] We wish to describe here an effective synthetic route to (−)-cucurbitine ((−)-**1**) and its thio analogue ((−)-**2**).

Our method included the 1,3-dipolar cycloaddition of the intermediary azomethine ylide (**4**)[4] and thiocarbonyl ylide (**13**)[5] with diethyl methylenemalonate (**5**),[6] asymmetric hydrolysis of the cycloadducts (**6**, **10** and **14**) to the chiral half esters ((+)-**7**, (−)-**11** and (−)-**15**) with pig liver esterase (PLE),[7] and conversion of the carboxyl group of the half esters to an amino group through Curtius rearrangement.[8]

Diethyl *N*-benzylpyrrolidine-3,3-dicarboxylate (**6**), a key starting compound, was synthesized by 1,3-dipolar cycloaddition of the intermediary azomethine ylide (**4**), derived from *N*-benzyl-*N*-(methoxymethyl)trimethylsilylmethylamine (**3**),[4] and diethyl methylenemalonate (**5**) in CH_2Cl_2 at 0 °C in 86% yield. Treatment of the 3,3-diester product (**6**) with PLE in 0.1 M phosphate buffer solution at 25 °C afforded the chiral half ester ((+)-**7**) in 63% yield. The enantiomeric excess (ee) of (+)-**7** was determined to be 10% by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analysis of ethyl methyl *N*-benzylpyrrolidine-3,3-dicarboxylate (**8**) using tris-[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato]europium (III) derivative as a shift reagent. The carboxyl group of (+)-**7** was then converted to the amino group with retention of configuration through Curtius rearrangement. Thus, the half ester ((+)-**7**) was treated with ethyl chloroformate in the presence of triethylamine, and then the reaction mixture was treated with sodium azide. After the usual work-up, the crude product was subjected to thermal rearrangement in benzene and treated with 20% HCl solution to afford (−)-**9**. The debenzylation of (−)-**9** with H_2/Pd–C gave (−)-**1**, natural cucurbitine, in 40% yield from the half ester. The infrared (IR) spectrum of the product was identical with that reported for an authentic sample.[3]

Diethyl pyrrolidine-3,3-dicarboxylate (**10**), prepared from **6** by catalytic hydrogenolysis, was subjected to similar asymmetric hydrolysis for 12 h to give the half ester ((−)-**11**).

$Me_3SiCH_2N(CH_2Ph)CH_2OMe$ (3) —CF_3COOH→ [CH_2–N(+)(CH₂Ph)–CH_2 ylide] (4) —$CH_2{=}C(CO_2Et)_2$ (5)→ 6 (N-CH_2Ph, CO_2Et, CO_2Et) —PLE→ (+)-7 (N-CH_2Ph, CO_2H, CO_2Et) —1) $ClCO_2Et$-Et_3N 2) NaN_3 3) C_6H_6 reflux 4) 20% HCl→ (−)-9 (N-CH_2Ph, NH_2, CO_2H) —Pd-C/H_2→ (−)-1 (N-H, NH_2, CO_2H)

6 —Pd-C/H_2→ 10 (N-H, CO_2Et, CO_2Et) —PLE→ (−)-11 (N-H, CO_2Et, CO_2H) —1) $ClCO_2Et$-Et_3N 2) NaN_3 3) C_6H_6 reflux 4) 20% HCl 5) Me_3SiI→ (+)-1 (N-H, CO_2H, NH_2)

$Me_3SiCH_2S(O)CH_2SiMe_3$ (12) —100°C in HMPA→ [CH_2–S(+)–CH_2 ylide] (13) —5→ 14 (S, CO_2Et, CO_2Et) —PLE→ (−)-15 (S, CO_2Et, CO_2H) —1) $ClCO_2Et$-Et_3N 2) NaN_3 3) C_6H_6 reflux 4) 20% HCl→ (−)-2 (S, CO_2H, NH_2) —Raney-Ni→ (+)-16 (H_2N–C(CO_2H)(CH_3)(C_2H_5))

Chart 1

Through Curtius rearrangement, (−)-**11** was converted to unnatural cucurbitine ((+)-**1**). The optical yield was determined to be 20% ee based on the optical rotation.

We also synthesized the thio analogue, 3-aminotetrahydrothiophene-3-carboxylic acid (**2**) from diethyl tetrahydrothiophene-3,3-dicarboxylate (**14**). The diester (**14**) was synthesized by 1,3-dipolar cycloaddition of the intermediary thiocarbonyl ylide (**13**), derived from bis(trimethylsilylmethyl)sulfoxide (**12**),[5] with diethyl methylenemalonate (**5**) in hexamethyltriamide phosphate (HMPA) at 100 °C, followed by hydrolysis with PLE at 25 °C in pH 8.0 phosphate buffer solution. A pH value of 8 was maintained by addition of 1 N NaOH. The half ester ((−)-**15**), obtained in 83% yield, was then treated successively with ethyl chloroformate at 0 °C in acetone and with sodium azide. The resulting azide derivative was subjected to thermal rearrangement in benzene followed by refluxing in 20% HCl solution to afford the thio analogue ((−)-**2**) in 85% yield. By direct desulfurization of (−)-**2** to isovaline ((+)-**16**)[9] with Raney-Ni, the absolute configuration and optical yield of (−)-**2** were determined to be 3-(*S*) and 6% ee, respectively.

Interestingly, in the asymmetric hydrolysis of the pyrrolidine (**10**) and the tetrahydrothiophene (**14**), the pro-*R* ester group was hydrolyzed, whereas in the case of the *N*-benzylpyrrolidine derivative (**6**), the pro-*S* ester group was cleaved. Although the optical yields, 6—20% ee, were not as high as expected,[10] we have established short routes for asymmetric syntheses of chiral cucurbitine and its thio analogue.

Experimental

All melting points are uncorrected. IR spectra were recorded with a JASCO A 202 infrared spectrophotometer. ^{1}H-NMR spectra were measured with a JEOL-90Q (90 MHz) FT-NMR spectrometer. ^{13}C-NMR spectra were measured with a JEOL-90Q (22.5 MHz) FT-NMR spectrometer. Chemical shifts are reported relative to internal tetramethylsilane or 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt hydrate and given as δ-values. Coupling constants are given 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. High-resolution mass spectra (MS) were recorded on a JEOL JMS-DX303 mass spectrometer.

Diethyl *N*-Benzylpyrrolidine-3,3-dicarboxylate (6)——A 1 mol/l solution of trifluoroacetic acid in CH_2Cl_2 (1 ml) was added to a mixture of *N*-benzyl-*N*-(methoxymethyl)trimethylsilylmethylamine (3)[4] (2.87 g, 12 mmol) and diethyl methylenemalonate (5) (1.72 g, 10 mmol) in CH_2Cl_2 (6 ml) in an ice-water bath. The reaction mixture was stirred for 2 h, washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography with benzene–tetrahydrofuran (THF) (30 : 1) to give an oil, 2.63 g (86%), bp 200 °C (bath temp.) (0.2 mmHg). IR ν_{max}^{neat} cm^{-1}: 1739 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.22 (6H, t, J=6.9 Hz, 2×CH_3), 2.43—2.66 (4H, m, NCH_2CH_2), 3.04 (2H, s, NCH_2C), 3.62 (2H, s, NCH_2Ph), 4.18 (4H, q, J=6.9 Hz, 2×OCH_2), 7.29 (5H, s, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 14.03 (q), 32.62 (t), 53.37 (t), 59.44 (t), 60.03 (t), 61.44 (s), 61.55 (t), 127.00 (d), 128.24 (d), 128.52 (d), 138.76 (s), 171.32 (s). *Anal.* Calcd for $C_{17}H_{23}NO_4$: C, 66.86; H, 7.59; N, 4.59. Found: C, 66.44; H, 7.53; N, 4.46.

(+)-*N*-Benzyl-3-ethoxycarbonylpyrrolidine-3-carboxylic Acid ((+)-7)——A mixture of PLE (300 unit) and **6** (1.155 g, 3.79 mmol) in 0.1 M phosphate buffer of pH 8.0 (30 ml) was stirred vigorously at 25 °C for 4 h. The pH value was adjusted to 10, then the solution was washed with CH_2Cl_2, neutralized with 3 N HCl, and concentrated under reduced pressure. $CHCl_3$ was added to the residue. Insoluble material was filtered off and the filtrate was concentrated under reduced pressure to give an oil, 660 mg (63%). $[\alpha]_D^{25}$ +5.03° (c=12.00, $CHCl_3$). IR ν_{max}^{neat} cm^{-1}: 1637, 1729 (CO), 3420 (NH^+). ^{1}H-NMR ($CDCl_3$) δ: 1.25 (3H, t, J=7.1 Hz, CH_3), 2.22—2.75 (2H, m, $N^+CH_2C\underline{H}_2$), 2.89—3.91 (4H, m, $CC\underline{H}_2N^+C\underline{H}_2CH_2$), 4.19 (2H, q, J=7.1 Hz, OCH_2), 4.23 (2H, s, N^+CH_2Ph), 7.26—7.64 (5H, m, C_6H_5), 10.29 (1H, s, COOH). ^{13}C-NMR ($CDCl_3$) δ: 13.87 (q), 31.75 (t), 53.48 (t), 56.57 (t), 58.62 (t), 61.55 (s), 62.70 (t), 129.28 (d), 129.82 (d), 130.74 (d), 137.41 (s), 169.20 (s), 171.53 (s). High-resolution MS (m/z): Calcd for $C_{14}H_{19}NO_2$ (M^+-CO_2): 233.1416. Found: 233.1408.

Ethyl Methyl *N*-Benzylpyrrolidine-3,3-dicarboxylate (8)——An ethereal solution of diazomethane was added in large excess to a solution of (+)-7 (60 mg, 0.22 mmol) in ether (5 ml). After concentration under reduced pressure, the residue was purified by silica gel chromatography with $CHCl_3$–THF (20 : 1) to give an oil, 45 mg (70%), bp 150—155 °C (bath temp.) (2 mmHg). IR ν_{max}^{neat} cm^{-1}: 1734 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, J=7.1 Hz, $CH_2C\underline{H}_3$), 2.34—2.50 (2H, m, $NCH_2C\underline{H}_2$), 2.60—2.77 (2H, m, $NC\underline{H}_2CH_2$), 3.05 (2H, s, NCH_2C), 3.63 (2H, s, $NC\underline{H}_2Ph$), 3.72 (3H, s, OCH_3), 4.19 (2H, q, J=7.1 Hz, OCH_2), 7.29 (5H, s, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 13.98 (q), 32.62 (t), 52.60 (q), 53.26 (t), 59.38 (t), 60.03 (t), 61.60 (t), 61.82 (s), 126.94 (d), 128.19 (d), 128.52 (d), 138.65 (s), 170.29 (s), 171.86 (s).

High-resolution MS (m/z): Calcd for $C_{16}H_{21}NO_4$ (M^+): 291.1471. Found: 291.1534.

(−)-*N*-Benzyl-3-aminopyrrolidine-3-carboxylic Acid ((−)-9)——A solution of triethylamine (238 mg, 2.35 mmol) in acetone (4 ml) was added to a solution of (−)-**7** (500 mg, 1.80 mmol) in acetone (4 ml). While maintaining the temperature at 0 °C, a solution of ethyl chloroformate (275 mg, 2.53 mmol) in acetone (1 ml) was added slowly. The mixture was stirred for 1 h at 0 °C and sodium azide (177 mg, 2.72 mmol) in water (1 ml) was added dropwise. The mixture was stirred at 0 °C for 1 h, then poured into ice-water (30 ml) and the whole was extracted with ether. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to leave an oil, which was dissolved in benzene (15 ml). The benzene solution was refluxed for 3 h and removal of the benzene under reduced pressure afforded an oil, which was dissolved in 20% HCl solution. This solution was refluxed for 2 h and concentrated under reduced pressure. The residue was purified with Amberlite IR-120B (H^+) ion-exchange resin column chromatography (5% aqueous ammonia) to give a white solid, 183 mg (44%), mp 200 °C (dec.). $[\alpha]_D^{20}-6.60°$ (c=1.00, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1679 (CO), 3456 (N^+H_3). ^{1}H-NMR (CD_3OD) δ: 1.70—3.89 (6H, m, $CH_2CH_2NCH_2C$), 3.68 (2H, s, $NC\underline{H}_2Ph$), 7.33 (5H, s, C_6H_5). ^{13}C-NMR (CD_3OD) δ: 37.28 (t), 54.18 (t), 60.41 (t), 64.53 (t), 66.43 (s), 128.24 (d), 129.33 (d), 129.98 (d), 139.41 (s), 177.60 (s). *Anal.* Calcd for $C_{12}H_{16}N_2O_2 \cdot 1/2H_2O$: C, 62.86; H, 7.47; N, 12.22. Found: C, 62.28; H, 7.41; N, 12.08.

(−)-Cucurbitine ((−)-1)——A mixture of (−)-**9** (100 mg, 0.44 mmol) and 5% Pd–C (50 mg) in methanol (20 ml) was stirred for 5 d at room temperature under a hydrogen atmosphere. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to give a solid, 52 mg (91%), mp 230 °C. $[\alpha]_D^{23}-2.12°$ (c=1.04, H_2O). [lit.,[2] 3-(S)-**1**, mp 260 °C (dec.), $[\alpha]_D^{27}-19.76°$ (c=9.31, H_2O)]. IR ν_{max}^{KBr} cm^{-1}: 1590 (CO), 3350 (N^+H_3). ^{1}H-NMR (CD_3OD) δ: 1.80—2.59 (2H, m, $NCH_2C\underline{H}_2$), 2.82—3.84 (4H, m, CH_2NCH_2). ^{13}C-NMR (CD_3OD) δ: 38.96 (t), 46.65 (t), 57.22 (t), 66.32 (s), 179.60 (s). High-resolution MS (m/z): Calcd for $C_5H_{10}N_2O_2$ (M^+): 130.0743. Found: 130.0768.

Diethyl Pyrrolidine-3,3-dicarboxylate (10)——A mixture of **6** (1.26 g, 4.13 mmol) and 5% Pd–C (400 mg) in ethanol (20 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, 785 mg (88%), bp 140—150 °C (bath temp.) (2 mmHg). IR ν_{max}^{neat} cm^{-1}: 1729 (CO), 3320, 3620 (NH). ^{1}H-NMR ($CDCl_3$) δ: 1.26 (6H, t, J=7.1 Hz, 2×CH_3), 2.33 (2H, t, J=7.0 Hz, $NCH_2C\underline{H}_2$), 2.21—2.50 (1H, br, NH), 3.04 (2H, t, J=7.0 Hz, $NC\underline{H}_2CH_2$), 3.36 (2H, s, NCH_2C), 4.20 (4H, q, J=7.1 Hz, 2×OCH_2). ^{13}C-NMR ($CDCl_3$) δ: 14.09 (q), 35.43 (t), 47.68 (t), 55.64 (t), 61.28 (s), 61.60 (t), 171.70 (s). High-resolution MS (m/z): Calcd for $C_{10}H_{17}NO_4$ (M^+): 215.1157. Found: 215.1132.

(−)-3-Ethoxycarbonylpyrrolidine-3-carboxylic Acid ((−)-11)——A mixture of PLE (200 unit) and **10** (431 mg, 2 mmol) in 0.1 M phosphate buffer of pH 8.0 (20 ml) was stirred vigorously at 25 °C for 12 h. The pH was adjusted to 10, then the solution was washed with ether, neutralized with 3 N HCl, and concentrated under reduced pressure. Ethanol was added to the residue. Insoluble material was filtered off and the filtrate was concentrated under reduced pressure to give an oil, 312 mg (83%). $[\alpha]_D^{23}-2.47°$ (c=4.70, EtOH). IR ν_{max}^{neat} cm^{-1}: 1621, 1734 (CO), 3400 (N^+H_2). ^{1}H-NMR (CD_3OD) δ: 1.27 (3H, t, J=7.1 Hz, CH_3), 2.09—2.54 (2H, m, $NCH_2C\underline{H}_2$), 3.18—3.72 (4H, m, $C\underline{H}_2NC\underline{H}_2CH_2$), 4.19 (2H, q, J=7.1 Hz, OCH_2). High-resolution MS (m/z): Calcd for $C_8H_{12}NO_4$ (M^+-H): 186.0766. Found: 186.0734.

(+)-Cucurbitine ((+)-1)——A solution of triethylamine (332 mg, 3.28 mmol) in acetone (1 ml) was added to a solution of (−)-**11** (235 mg, 1.26 mmol) in acetone (2 ml). While maintaining the temperature at 0 °C, a solution of ethyl chloroformate (383 mg, 3.53 mmol) in acetone (1 ml) was added slowly. The mixture was stirred for 30 min at 0 °C and then sodium azide (123 mg, 1.89 mmol) in water (1 ml) was added dropwise. The mixture was stirred at 0 °C for 2 h, then poured into ice-water (30 ml) and the mixture was extracted with ether. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to leave an oil, which was dissolved in benzene (10 ml). The benzene solution was refluxed for 2 h and removal of the benzene under reduced pressure afforded an oil, which was dissolved in 20% HCl solution (5 ml). The solution was refluxed for 2 h and concentrated under reduced pressure to give an oil. This was purified by Amberlite IR-120B (H^+) ion-exchange resin column chromatography (5% aqueous ammonia) to give a white solid, which was dissolved in CH_3CN (4 ml). Trimethylsilyl iodide (152 mg, 0.76 mmol) was added to the solution under a nitrogen atmosphere and the mixture was refluxed for 24 h. After removal of the CH_3CN under reduced pressure, the residue was purified by Amberlite IR-120B (H^+) ion-exchange resin chromatography (5% aqueous ammonia) to give a solid, 31 mg (22%), mp 230 °C. $[\alpha]_D^{22}+3.94°$ (c=0.16, H_2O). The IR, ^{1}H-NMR, and ^{13}C-NMR spectral data were in agreement with those of (−)-**1**.

of (−)-1

Diethyl Tetrahydrothiophene-3,3-dicarboxylate (14)——A mixture of bis(trimethylsilylmethyl)sulfoxide (**12**)[5] (6.67 g, 30 mmol) and **5** (3.44 g, 20 mmol) in HMPA (4 ml) was added to HMPA (4 ml) at 100 °C and the mixture was stirred for 15 min at 100 °C. After removal of HMPA by silica gel chromatography with hexane–THF (20 : 1), the obtained oil was purified by silica gel chromatography with benzene to give a colorless oil, 928 mg (20%), bp 140 °C (bath temp.) (2 mmHg). IR ν_{max}^{neat} cm^{-1}: 1738 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.22 (6H, t, J=7.2 Hz, 2×CH_3), 2.48 (2H, t, J=6.8 Hz, $SCH_2C\underline{H}_2$), 2.91 (2H, t, J=6.8 Hz, $SC\underline{H}_2CH_2$), 3.32 (2H, s, SCH_2C), 4.18 (4H, q, J=7.2 Hz, 2×OCH_2). ^{13}C-NMR ($CDCl_3$) δ: 13.98 (q), 29.80 (t), 36.73 (t), 37.66 (t), 61.93 (t), 64.31 (s), 169.80 (s). *Anal.* Calcd for $C_{10}H_{16}O_4S$: C, 51.71; H, 6.94. Found: C, 51.33; H, 7.10.

(−)-3-Ethoxycarbonyltetrahydrothiophene-3-carboxylic Acid ((−)-15)——A mixture of PLE (200 unit) and **14**

(232 mg, 1 mmol) in 0.1 M phosphate buffer of pH 8.0 (20 ml) was stirred vigorously at 25 °C for 4 h. The pH was adjusted to 10, and the mixture was washed with CH_2Cl_2. The pH was adjusted to 2 with 10% HCl solution and the solution was extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give an oil, 170 mg (83%). $[\alpha]_D^{22}$ −0.26° (c=3.40, $CHCl_3$). IR ν_{max}^{neat} cm^{-1}: 1728, 1737 (CO), 3216 (COOH). ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, t, J=7.1 Hz, CH_3), 2.57 (2H, t, J=6.1 Hz, $SCH_2C\underline{H}_2$), 2.98 (2H, t, J=6.1 Hz, $SC\underline{H}_2CH_2$), 3.39 (2H, s, SCH_2C), 4.26 (2H, q, J=7.1 Hz, OCH_2), 9.28—9.52 (1H, br, COOH). ^{13}C-NMR ($CDCl_3$) δ: 13.92 (q), 29.75 (t), 36.73 (t), 37.66 (t), 62.39 (t), 64.26 (s), 169.31 (s), 175.38 (s). High-resolution MS (m/z): Calcd for $C_8H_{12}O_4S$ (M^+): 204.0456. Found: 204.0435.

(−)-3-Aminotetrahydrothiophene-3-carboxylic Acid ((−)-2)——A solution of triethylamine (101 mg, 1.00 mmol) in acetone (2 ml) was added to a solution of (−)-**15** (170 mg, 0.83 mmol) in acetone (3 ml). While maintaining the temperature at 0 °C, a solution of ethyl chloroformate (117 mg, 1.08 mmol) in acetone (1 ml) was added slowly. The mixture was stirred for 30 min at 0 °C and then sodium azide (81 mg, 1.25 mmol) in water (1 ml) was added dropwise. The whole was stirred for 3 h, then poured into ice-water (20 ml) and this mixture was extracted with ether. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to leave an oil, which was dissolved in benzene (10 ml). The benzene solution was refluxed for 1 h and removal of the benzene under reduced pressure afforded an oil, which was dissolved in 20% HCl solution (5 ml). This solution was refluxed for 2 h and concentrated under reduced pressure. The residue was purified by Amberlite IR-120B (H^+) ion-exchange resin column chromatography (5% aqueous ammonia) to give a white solid, 104 mg, (76%), mp 207—209 °C. $[\alpha]_D^{25}$ −6.67° (c=1.32, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1680 (CO), 3072, 3500 (N^+H_3). ^{1}H-NMR (D_2O) δ: 2.36—2.54 (2H, m, $SCH_2C\underline{H}_2$), 2.70—3.26 (2H, m, $SC\underline{H}_2CH_2$), 3.03 (1H, d, J=12.4 Hz, $SC\underline{H}_AHC$), 3.40 (1H, d, J=12.4 Hz, $SC\underline{H}_BHC$). ^{13}C-NMR (D_2O) δ: 31.59 (t), 41.67 (t), 41.83 (t), 72.49 (s), 176.63 (s). *Anal.* Calcd for $C_5H_9NO_2S\ H_2O$: C, 36.35; H, 6.71; N, 8.48. Found: C, 36.16; H, 6.57; N, 8.40.

(+)-Isovaline ((+)-16)——A mixture of (−)-**2** (92 mg, 0.56 mmol) and Raney-Ni W6 (1 g) in water (4 ml) was stirred overnight at room temperature. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to afford a solid, which was purified by Amberlite IR-120B (H^+) ion-exchange resin column chromatography (5% aqueous ammonia) to give a white solid, 25 mg (38%). $[\alpha]_D^{24}$ +1.60° (c=0.50, AcOH). [lit,[9] (S)-**16**, $[\alpha]_D^{25}$ +26.3° (c=2, AcOH)]. IR ν_{max}^{KBr} cm^{-1}: 1585 (CO), 3450 (N^+H_3). ^{1}H-NMR (D_2O) δ: 0.91 (3H, t, J=7.5 Hz, $C\underline{H}_3CH_2$), 1.45 (3H, s, CH_3), 1.58—1.89 (2H, m, $CH_3C\underline{H}_2$).

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A Simple One-Pot Synthesis of *tert*-Butyldimethylsilyl-Protected Cyanohydrins

RYUJI YONEDA, HIDEKI HISAKAWA, SHINYA HARUSAWA, and TAKUSHI KURIHARA*

Osaka University of Pharmaceutical Sciences, 2-10-65, Kawai, Matsubara, Osaka 580, Japan

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A direct conversion of a variety of aldehydes to the corresponding *tert*-butyldimethylsilyl protected cyanohydrins is described, by using *tert*-butyldimethylsilyl chloride and lithium cyanide.

Keywords——*tert*-butyldimethylsilyl chloride; lithium cyanide; *tert*-butyldimethylsilyl protected cyanohydrin; one-pot synthesis; non-aqueous cyanation; non-catalyzed cyanation

Trimethylsilyl (TMS) protected cyanohydrins, which are well known to be quite useful intermediates in organic synthesis,[1] are in general prepared from aldehydes and ketones by thermal and catalyzed addition of TMS cyanide (TMSCN) or by reaction with TMSCl and potassium cyanide (KCN) in the presence of 18-crown-6 or Lewis acids such as ZnX_2. In order to overcome the problem of the labile nature of the TMS group,[2] Corey *et al.* prepared *tert*-butyldimethylsilyl (TBDMS)-protected cyanohydrins of ketones, which are stable during purification by column chromatography as well as hydrolysis (30% H_2O_2, K_2CO_3 in MeOH) of the CN group.[3] TBDMS-protected cyanohydrins are usually prepared by treatment of a ketone or aldehyde with an excess of TBDMSCN in the presence of crown ether[3] or ZnI_2.[4] However, the preparation and purification of TBDMSCN is somewhat troublesome.[3,5] In 1985, Cava and co-workers reported a simple high yielding procedure for the direct conversion of aldehydes to TBDMS protected cyanohydrins by using TBDMSCl (1.2 eq) and KCN (4 eq) in the presence of ZnI_2 as a catalyst.[6] Recently we reported a simple one-pot process for the high yielding conversion of a variety of carbonyl compounds into TMS protected cyanohydrins by using TMSCl and lithium cyanide (LiCN) without any catalyst.[7] The greater reactivity of LiCN over sodium cyanide, KCN, and silver cyanide is due to its greater solubility in many organic solvents and the higher charge density of the lithium ion.[8] As a continuation of our studies on the utilization of LiCN[9] as a cyanation agent,[10] we describe here another method for the synthesis of TBDMS protected cyanohydrins.

The reaction was performed simply by using two equivalents of TBDMSCl and three equivalents of LiCN at room temperature in dry tetrahydrofuran (THF), and the resulting crude product was purified by column chromatography on silica-gel. For comparison, when we ran this reaction with 1-phenylsulfonylpyrrole-2-carbaldehyde (**1a**), the TBDMS protected cyanohydrin (**2a**) was obtained in 98% yield, slightly higher than that (96% yield in 12 h) reported by Cava *et al.*[6] A number of aldehydes, including aliphatic and aromatic or heteroaromatic aldehydes, were converted to TBDMS-protected cyanohydrins (**2b**—**h**) in excellent yields. The results are summarized in the table.

As mentioned by Cava *et al.*, the ketones were considerably less reactive with TBDMSCl, KCN, and ZnI_2, and gave the TBDMS enol ethers as by-products. Therefore, their method was limited to aldehydes. On the other hand, although ketones could not be converted satisfactorily to the desired silylated cyanohydrins, our procedure exceptionally led cyclo-

TBDMSCl/LiCN

in THF at r.t.

1 2

	R	R′		R	R′		R	R′
a	SO_2Ph	H	b		H	c		H
d	NO_2	H	e		H	f	Tos	H
g		H	h	$-(CH_2)_5-$			Tos = *p*-toluenesulfonyl	

Chart 1

Table I. *tert*-Butyldimethylsilyl-Protected Cyanohydrins (2)

Compd. No.	Reaction time (h)	Yield[a] (%)	^{1}H-NMR (δ in $CDCl_3$ from TMS)
2a[b]	6	97	0.08 and 0.15 (each 3H, each s, $2\times CH_3$), 0.89 [9H, s, $C(CH_3)_3$], 6.03 (1H, s, CHCN), 6.33 (1H, t, J=3.3 Hz, 4-H on pyrrole ring), 6.64 (1H, m, 3-H on pyrrole ring), 7.25 (1H, m, 5-H on pyrrole ring), 7.5—7.8 (5H, m, Ar-H)
2b[b]	1	91	0.15 and 0.23 (each 3H, each s, $2\times CH_3$), 0.94 [9H, s, $C(CH_3)_3$], 5.52 (1H, s, CHCN), 7.35—7.50 (5H, m, Ar-H)
2c[b]	1	98	0.12 and 0.20 (each 3H, each s, $2\times CH_3$), 0.92 [9H, s, $C(CH_3)_3$], 5.38 (1H, s, CHCN), 5.98 (2H, s, OCH_2O), 6.79 (1H, d, J=8 Hz, 6-H), 6.87—6.95 (2H, m, Ar-H)
2d	1	88	0.21 and 0.29 (each 3H, each s, $2\times CH_3$), 0.97 [9H, s, $C(CH_3)_3$], 5.62 (1H, s, CHCN), 7.6—7.9 (2H, m, 5- and 6-H), 8.2—8.4 (2H, m, 2- and 4-H)
2e	0.5	82	0.18 and 0.27 (each 3H, each s, $2\times CH_3$), 0.97 [9H, s, $C(CH_3)_3$], 5.62 (1H, s, CHCN), 7.3—7.9 (3H, m, Ar-H), 8.60 (1H, m, 6-H)
2f	48	78	0.12 and 0.20 (each 3H, each s, $2\times CH_3$), 0.89 [9H, s, $C(CH_3)_3$], 2.32 (3H, s, Ar-CH_3), 5.71 (1H, s, CHCN), 7.2—8.0 (9H, m, Ar-H)
2g	7	80	0.12 and 0.18 (each 3H, each s, $2\times CH_3$), 0.92 [9H, s, $C(CH_3)_3$], 1.0—1.9 (11H, m, cyclohexyl-H), 4.17 (1H, d, J=5.9 Hz, CHCN)
2h	70	100	0.23 (6H, s, $2\times CH_3$), 0.90 [9H, s, $C(CH_3)_3$], 1.3—2.1 (10H, m, cyclohexyl-H)

a) Yields are based on isolation of chromatographically purified products. *b*) The ^{1}H-NMR spectrum was identical with that reported by Cava *et al.*[6]

hexanone[11] to the TBDMS-protected cyanohydrin (**2h**) in very good yield, though the reaction required a long time. Our reaction probably involves the initial formation of the lithium cyanoalkoxide and subsequent substitution of TBDMSCl.

We consider that our procedure is superior to the previous one,[6] in that the use of a Lewis acid is unnecessary.

Experimental

The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian XL-300 (300 MHz) spectrometer using tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a Hitachi M-80 spectrometer.

General Procedure for the Preparation of *tert*-Butyldimethylsilyl-Protected Cyanohydrins (2a—h)——LiCN (297 mg, 9 mmol) was added to a magnetically stirred solution of a carbonyl compound (3 mmol) and TBDMSCl (904 mg, 6 mmol) in dry THF (10 ml) at room temperature under a stream of nitrogen. The reaction was monitored by thin layer chromatography (TLC) [silica-gel (Merck Art 5715), benzene]. After the starting material had been consumed, the THF was evaporated off. The residue was partitioned between water (10 ml) and benzene–EtOAc (1:1) (50 ml). The organic layer was separated, washed with water (2 × 10 ml), and dried with Na_2SO_4. After concentration of the organic layer, the residue was chromatographed on a silica-gel (Merck Art 7734) column, with benzene as the eluent.

α-[(*tert*-Butyldimethylsilyl)oxy]-1-phenylsulfonyl-2-pyrroloacetonitrile (**2a**): A colorless oil. MS *m/z*: 376 (M^+).

α-[(*tert*-Butyldimethylsilyl)oxy]phenylacetonitrile (**2b**): A colorless oil. MS *m/z*: 247 (M^+).

α-[(*tert*-Butyldimethylsilyl)oxy]-3,4-methylenedioxyphenylacetonitrile (**2c**): A colorless oil. MS *m/z*: 259 (M^+).

α-[(*tert*-Butyldimethylsilyl)oxy]-3-nitro-1-phenylacetonitrile (**2d**): A colorless oil, *Rf* 0.54. High resolution MS (HRMS) Calcd for $C_{14}H_{20}N_2O_3Si$: 292.124. Found: 292.124.

α-[(*tert*-Butyldimethylsilyl)oxy]-2-pyridylacetonitrile (**2e**): A colorless oil, *Rf* 0.14. HRMS Calcd for $C_{13}H_{20}N_2OSi$: 248.134. Found: 248.134.

α-[(*tert*-Butyldimethylsilyl)oxy]-1-*p*-toluenesulfonyl-3-indolylacetonitrile (**2f**): A colorless oil, *Rf* 0.61. HRMS Calcd for $C_{23}H_{28}N_2O_3SSi$: 440.159. Found: 440.159.

α-[(*tert*-Butyldimethylsilyl)oxy]cyclohexylacetonitrile (**2g**): A colorless oil, *Rf* 0.81. HRMS Calcd for $C_{14}H_{27}NOSi$: 253.186. Found: 253.186.

1-[(*tert*-Butyldimethylsilyl)oxy]-1-cyanocyclohexane (**2h**): A colorless oil, *Rf* 0.78. HRMS Calcd for $C_{13}H_{25}NOSi$: 239.170. Found: 239.170.

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11) According to the method of Cava *et al.*,[6] **2h** was obtained in only 8% yield.

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Amino Acids and Peptides. XVII.[1,2] Synthesis of a Tridecapeptide Corresponding to the Sequence 165—177 of T-Kininogen (Tryptic Peptide) Containing Gln–Val–Val–Ala–Gly Sequence and the Relationship between Structure and Effect on Thiol Proteinase

NAOKI TENO,[a] SATOSHI TSUBOI,[a] YOSHIO OKADA,*,[a]
NORIO ITOH[b] and HIROSHI OKAMOTO[b]

Department of Medicinal Chemistry[a] and Pharmacology,[b] Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Nishi-ku, Kobe 673, Japan

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The tridecapeptide corresponding to the sequence 165—177 of T-kininogen (tryptic peptide) (I) containing Gln–Val–Val–Ala–Gly sequence was synthesized by a conventional solution method and its effect on thiol proteinase was examined. Although Z–Gln–Val–Val–Ala–Gly–OMe showed inhibitory activity towards papain and protective activity against T-kininogen-induced inhibition of papain, the tryptic peptide obtained did not exhibit any effect on thiol proteinase. In order to study the relationship between structure and effect on thiol proteinase, several compounds (II—VI) modified with various groups at the N-terminus of the Gln–Val–Val–Ala–Gly sequence were synthesized. Peptides V and VI, which have an aromatic ring at the N-terminus, exhibited weak inhibitory and significant protective activities. A small peptide such as Gln–Val–Val–Ala–Gly might be better able to approach papain than the tryptic peptide (I), and the aromatic ring associated with Gln–Val–Val–Ala–Gly at the N-terminus apparently strengthened the binding ability of the peptide to papain.

Keywords——thiol proteinase inhibitor; T-kininogen; tryptic peptide; chemical synthesis; inhibitory activity; protective activity; papain; structure–activity relationship; Gln–Val–Val–Ala–Gly sequence

Thiol proteinase inhibitors with high and low molecular weights have been isolated from plasma and various tissues.[3,4] These inhibitors inactivate many thiol proteinases, including cathepsins, ficin, papain and calpain.

Recently, Ohkubo *et al.*[5] reported that one of the thiol proteinase inhibitors (α_2-TPI) in human plasma is identical with low-molecular-weight kininogen in bovine plasma by analyzing the base sequence of cDNA for α_2-TPI. Takio *et al.*[4] concluded that the structures of several mammalian low-molecular-weight thiol proteinase inhibitors were highly homologous. It is also interesting that there is a fairly conservative common amino acid sequence, Gln–Val–Val–Ala–Gly,[5] which might be one of the reactive sites of endogenous thiol proteinase inhibitors.

Previously, we synthesized Z–Gln–Val–Val–Ala–Gly–OMe and its derivatives and examined their effects on the enzymatic activity of papain.[6,7] One interesting finding with these derivatives was that Z–Gln–Val–Val–Ala–Gly–OMe weakly inhibited papain activity, and further, protected papain from inhibition by the thiol proteinase inhibitor such as T-kininogen.[6,7] It was deduced that the compound bound with some part of papain in competition with T-kininogen. Studies on the relationship between the structures of these small peptides and their effects on thiol proteinases should provide useful information on the

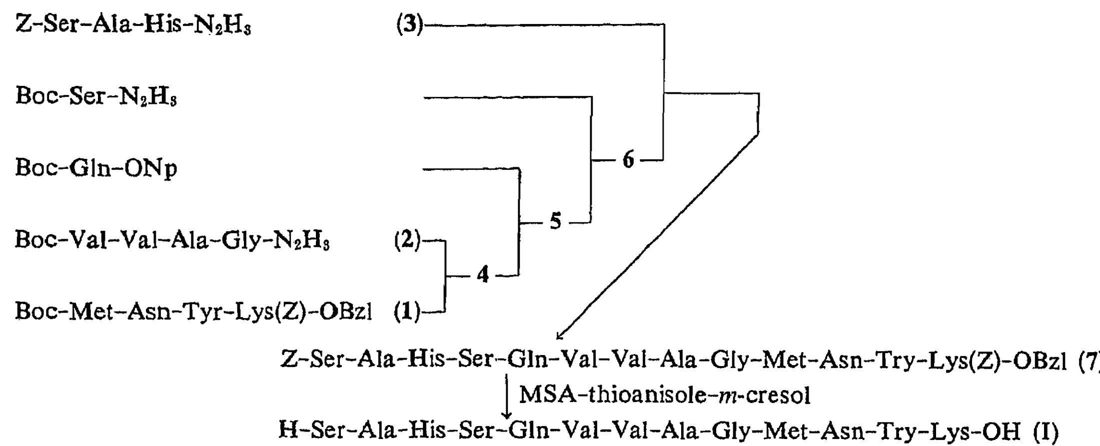

Fig. 1. Synthetic Scheme for Tridecapeptide Corresponding to the Sequence 165—177 of T-Kininogen (Tryptic Peptide) (I)

mechanisms of action of thiol proteinases. In the present paper, we describe the synthesis of T-kininogen tryptic peptide (T-kininogen 165—177, tridecapeptide (I)) (Fig. 1) containing the Gln–Val–Val–Ala–Gly sequence, its effect on papain, and the relationship between structure and effect on thiol proteinase.

As illustrated in Fig. 1, in combination with the TFA-labile Boc group for N^{α}-protection, amino acid derivatives Z–Ser–OH and H–Lys(Z)–OBzl bearing protecting groups removable by MSA–thioanisole–*m*-cresol[8] were employed for the synthesis.

The C-terminal fragment (1) was prepared as follows. Boc–Asn–ONp[9] was coupled with H–Tyr–OMe,[10] followed by hydrazine hydrate treatment to give Boc–Asn–Tyr–N_2H_3. Boc–Asn–Tyr–N_2H_3 and H–Lys(Z)–OBzl[11] were coupled by the azide method to yield Boc–Asn–Tyr–Lys(Z)–OBzl. The Boc group of Boc–Asn–Tyr–Lys(Z)–OBzl was removed by TFA–anisole, yielding the corresponding amine, which was coupled with Boc–Met–ONp[12] to afford Boc–Met–Asn–Tyr–Lys(Z)–OBzl (1).

Boc–Val–Val–Ala–Gly–OMe was prepared by stepwise condensation using the mixed anhydride method, starting with H–Gly–OMe. Boc–Val–Val–Ala–Gly–OMe thus obtained was exposed to hydrazine hydrate in MeOH to give the corresponding hydrazide, Boc–Val–Val–Ala–Gly–N_2H_3 (2).

Z–Ser–Ala–OMe[13] was exposed to hydrazine hydrate in EtOH to give the corresponding hydrazide. Z–Ser–Ala–N_2H_3 and H–His–OMe were then coupled by the azide method to give Z–Ser–Ala–His–OMe, which was converted to the desired hydrazide (3) in the usual manner.

The fragments obtained above were assembled starting with the C-terminal fragment, H–Met–Asn–Tyr–Lys(Z)–OBzl, as shown in Fig. 1. The protected tridecapeptide (7), sequence 165—177 of rat T-kininogen repeat 1, thus obtained was purified by washing with 10% EtOH.

At the final step, deprotection and subsequent purification were carried out according to the procedure described below. The protected compound was treated with MSA in the presence of thioanisole and *m*-cresol in an ice-bath for 30 min and at room temperature for 60 min.[14,15] After precipitation of the deprotected peptide with ether, the product was converted to the corresponding acetate form by treatment with Amberlite IRA 45 (acetate form). The pH of the solution was adjusted with 1 M NH_4OH to 8.0 in order to reverse the N→O shift at Ser[16] and after 30 min, readjusted to 6.5 with 1 N AcOH. The solution was lyophilized to give a hygroscopic powder, which was dissolved in H_2O and reduced with dithiothreitol. The final product was purified by gel-filtration on Sephadex G-25 using 3% AcOH as an eluant. The major peak obtained exhibited a single spot on thin-layer chromatography (TLC), but showed small side peaks on high-performance liquid chromatog-

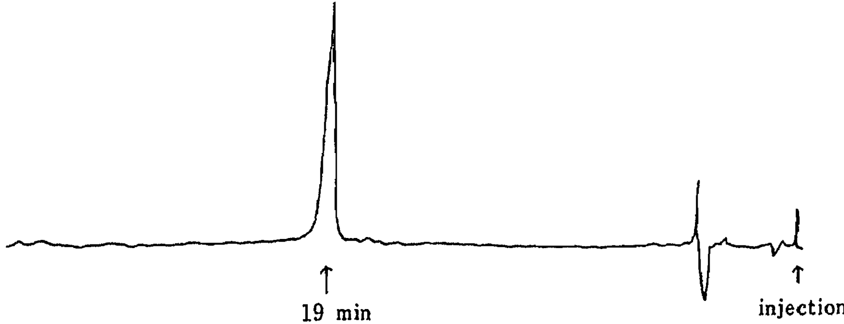

Fig. 2. HPLC Pattern of Tridecapeptide Corresponding to the Sequence 165—177 of T-Kininogen (Tryptic Peptide) (I)

Column, YMC R-ODS-5 (4.6×250 mm); absorbance, 220 nm; eluent, gradient elution with CH_3CN (15—30% in 30 min) in 0.1% TFA; flow rate, 1 ml/min; chart speed, 5 mm/min.

TABLE I. Effects of Tryptic Peptide (I) and X–Gln–Val–Val–Ala–Gly–OMe (II—VII) on the Enzymatic Activity of Papain and on the Inhibitory Activity of T-Kininogen toward Papain

	Inhibition of papain (%)[a]	Protection of papain from T-kininogen-induced inhibition[b]
I	0	1.0
II (X=Boc)	2.3	1.0
III (X=H)	0	1.0
IV (X=Ac)	4.1	1.0
V (X=Benzoyl)	7.0	1.9
VI (X=β-Naphthoyl)	9.3	2.1
VII (X=Z)[c]	20.5	5.8

a) The inhibitory effects of the peptides were determined by adding the peptides (0.18 mM) to the assay medium. The values represent the means of 4 experiments. *b*) The protective effects of peptides on inhibitory activity of T-kininogen toward papain were calculated as follows:

$$\text{factor} = \frac{IC_{50} \text{ of T-kininogen in the presence of peptide}}{IC_{50} \text{ of T-kininogen in the absence of peptide}}.$$

c) See references 6 and 7.

raphy (HPLC). Preparative reverse-phase HPLC on a YMC D-ODS-5 column was effective for removing these minor peaks. The tridecapeptide (I) thus obtained exhibited a single peak on HPLC as shwon in Fig. 2, and the results of amino acid analysis were in good agreement with the theoretically expected values.

The inhibitory activity of I against papain and its protective effect against thiol proteinase inhibitor-induced inhibition of papain were determined with a synthetic substrate, Bz–Arg–βNA[17] by means of he techniques previously described.[6,7]

The tridecapeptide (I) did not show any inhibitory activity against papain or protective activity against T-kininogen-induced inhibition, although it contains the Gln–Val–Val–Ala–Gly sequence. It is not clear why Z–Gln–Val–Val–Ala–Gly–OMe could inhibit papain activity slightly and protect papain from T-kininogen-induced inhibition,[6,7] whereas peptide I could not. Possibly the Gln–Val–Val–Ala–Gly moiety in the tridecapeptide molecule (I) is buried in the molecule of I, or the N-terminal part and/or C-terminal part of the tridecapeptide (I) block the access of the Gln–Val–Val–Ala–Gly sequence to the binding site on papain.

In order to examine these possibilities, we prepared Gln–Val–Val–Ala–Gly derivatives substituted at the N-terminus (II—VI). Their effects on papain were examined and the results are summarized in Table I. The results show that peptides II, IV, V and VI weakly inhibited the amidolytic action of papain toward Bz–Arg–βNA, but peptide III did not. This demonstrates that the N-terminal protecting groups are necessary for manifestation of inhibitory activity. With regard to protective activity against T-kininogen-induced inhibition of papain, only peptides V and VI, which have an aromatic ring at the N-terminus, exhibited the activity.

In conclusion, although the tryptic peptide obtained did not have any effect on papain, some Gln–Val–Val–Ala–Gly derivatives exhibited inhibitory and protective effects. Thus, a small peptide such as Gln–Val–Val–Ala–Gly might be better able to approach the enzyme and bind with it than I, and the presence of an aromatic ring at the N-terminus of the pentapeptide strengthens the binding ability of the peptide.

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 (110 °C, 18 h, 6 N HCl) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). For column chromatography, a Toyo SF-160K fraction collector was used. HPLC was conducted with a Waters 600 model. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and water (89 : 10 : 1), $CHCl_3$, MeOH and water (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and water (4 : 1 : 5, upper phase), *n*-BuOH, AcOH, pyridine and water (4 : 1 : 1 : 2) and *n*-BuOH, AcOH, pyridine and water (1 : 1 : 1 : 1), respectively.

Boc–Asn–Tyr–OMe——Boc–Asn–ONp (4.9 g) and H–Tyr–OMe (prepared from 3.1 g of H–Tyr–OMe·HCl and 1.8 ml of Et_3N) were dissolved in DMF (20 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 2.6 g (48.9%), mp 179—180 °C, $[\alpha]_D^{25}-5.3°$ (c= 0.3, DMF), Rf^1 0.28. *Anal.* Calcd for $C_{19}H_{27}N_3O_7$: C, 55.8; H, 6.60; N, 10.3. Found: C, 55.9; H, 6.80; N, 10.0.

Boc–Asn–Tyr–N_2H_3——Hydrazine hydrate (90%, 0.34 ml) was added to a solution of Boc–Asn–Tyr–OMe (1.0 g) in MeOH (15 ml). The mixture was stored at room temperature overnight. The crystals that formed were collected by filtration and recrystallized from EtOH, yield 0.72 g (88.0%), mp 210—213 °C, $[\alpha]_D^{25}-25.0°$ (c=0.2, DMF), Rf^3 0.46. *Anal.* Calcd for $C_{18}H_{27}N_5O_6\cdot 1/2H_2O$: C, 51.7; H, 6.69; N, 15.7. Found: C, 51.9; H, 6.75; N, 15.5.

Boc–Asn–Tyr–Lys(Z)–OBzl——Boc–Asn–Tyr–N_3 (prepared from 1.5 g of Boc–Asn–Tyr–N_2H_3 and 0.42 ml of isopentyl nitrite as usual) in DMF (25 ml) was added to a solution of H–Lys(Z)–OBzl (prepared from 2.4 g of H–Lys(Z)–OBzl·TosOH and 0.63 ml of Et_3N) in DMF (15 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from EtOH, yield 2.1 g (93.7%), mp 184—186 °C, $[\alpha]_D^{25}-23.2°$ (c=0.4, DMF), Rf^2 0.28, Rf^3 0.85. *Anal.* Calcd for $C_{39}H_{49}N_5O_{10}\cdot H_2O$: C, 61.2; H, 6.66; N, 9.15. Found: C, 61.1; H, 6.31; N, 9.12.

Boc–Met–Asn–Tyr–Lys(Z)–OBzl (1)——The title compound was prepared from Boc–Met–ONp (0.21 g) and H–Asn–Tyr–Lys(Z)–OBzl·TFA (prepared from 0.43 g Boc–Asn–Tyr–Lys(Z)–OBzl and 0.42 ml of TFA containing 0.12 ml of anisole) and recrystallized from AcOEt, yield 0.40 g (79.9%), mp 153—154 °C, $[\alpha]_D^{25}-26.4°$ (c=0.2, DMF), Rf^2 0.51, Rf^3 0.71. *Anal.* Calcd for $C_{44}H_{58}N_6O_{11}S\cdot H_2O$: C, 59.0; H, 6.69; N, 9.37. Found: C, 58.9; H, 6.58; N, 9.35. Amino acid ratios in a 6 N HCl hydrolysate: Met 0.81, Asp 1.05, Tyr 0.84, Lys 1.00 (average recovery 81.9%).

Boc–Val–Val–Ala–Gly–OMe——A mixed anhydride (prepared from 2.4 g of Boc–Val–OH and 1.5 ml of isobutyl chloroformate as usual) in THF (100 ml) was added to an ice-cold solution of H–Val–Ala–Gly–OMe·HCl (prepared from 2.9 g of Z–Val–Ala–Gly–OMe[7] by catalytic hydrogenation). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 2.6 g (76.7%), mp 220—222 °C, $[\alpha]_D^{25}-21.1°$ (c=0.5, DMF), Rf^1 0.55, Rf^2 0.65. *Anal.* Calcd for $C_{21}H_{38}N_4O_7$: C, 55.1; H, 8.29; N, 12.2. Found: C, 54.8; H, 8.54; N, 12.2.

Boc–Val–Val–Ala–Gly–N_2H_3 (2)——Reaction and work-up for Boc–Asn–Tyr–N_2H_3 with Boc–Val–Val–Ala–

Gly–OMe (0.78 g) and hydrazine hydrate (90%, 0.28 ml) gave the title compound, yield 0.46 g (58.8%), mp 228—231 °C, $[\alpha]_D^{25}$ −28.3° (c=0.4, DMSO), Rf^1 0.17, Rf^3 0.69. *Anal.* Calcd for $C_{20}H_{38}N_6O_6 \cdot 1/4H_2O$: C, 51.9; H, 8.32; N, 18.2. Found: C, 51.9; H, 8.37; N, 18.0. Amino acid ratios in a 6 N HCl hydrolysate for 72 h[7]: Val 1.52, Ala 1.08, Gly 1.00 (average recovery 88.5%).

Z–Ser–Ala–N_2H_3——Reaction and work-up for Boc–Asn–Tyr–N_2H_3 with Z–Ser–Ala–OMe (5.0 g) and hydrazine hydrate (90%, 2.6 ml) gave the title compound, yield 4.6 g (95.8%), mp 218—223 °C, $[\alpha]_D^{25}$ −5.2° (c=1.0, DMF), Rf^3 0.25, Rf^4 0.60. *Anal.* Calcd for $C_{16}H_{20}N_4O_5 \cdot H_2O$: C, 52.5; H, 6.05; N, 15.3. Found: C, 52.6; H, 6.12; N, 15.2.

Z–Ser–Ala–His–OMe——The title compound was obtained from Z–Ser–Ala–N_3 (prepared from 0.63 g of Z–Ser–Ala–N_2H_3 and 0.28 ml of isopentyl nitrite as usual) and H–His–OMe (prepared from 0.5 g of H–His–OMe·2HCl and 0.56 ml of Et_3N) in the same way as described for the synthesis of Boc–Asn–Tyr–Lys(Z)–OBzl, yield 0.54 g (57.9%), mp 179—182 °C, $[\alpha]_D^{25}$ −0.7° (c=0.9, DMF), Rf^4 0.19, Rf^5 0.60. *Anal.* Calcd for $C_{21}H_{27}N_5O_7$: C, 54.7; H, 5.91; N, 15.2. Found: C, 54.6; H, 5.89; N, 15.1.

Z–Ser–Ala–His–N_2H_3 (3)——Reaction and work-up for Boc–Asn–Tyr–N_2H_3 with Z–Ser–Ala–His–OMe (1.3 g) and hydrazine hydrate (90%, 0.47 ml) gave the title compound, yield 0.69 g (57.5%), mp 171—175 °C, $[\alpha]_D^{25}$ −2.3° (c=0.3, DMF), Rf^4 0.18, Rf^5 0.48. *Anal.* Calcd for $C_{20}H_{27}N_7O_6 \cdot 3/4H_2O$: C, 50.6; H, 6.06; N, 20.6. Found: C, 50.8; H, 5.85; N, 20.4. Amino acid ratios in a 6 N HCl hydrolysate: Ser 1.01, Ala 1.00, His 1.00 (average recovery 76.0%).

Boc–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl (4)——The title compound was obtained from Boc–Val–Val–Ala–Gly–N_3 (prepared from 0.14 g of **2** and 0.04 ml of isopentyl nitrite as usual) and H–Met–Asn–Tyr–Lys(Z)–OBzl·TFA (prepared from 0.14 g of **1** and 0.62 ml of TFA containing 0.1 ml of 3,5-dimethylanisole and 0.02 ml of 1,2-ethanedithiol)[18] in the same way as described for the synthesis of Boc–Asn–Tyr–Lys(Z)–OBzl, yield 0.14 g (77.5%), mp 220—223 °C, $[\alpha]_D^{25}$ −94.3° (c=0.1, DMF), Rf^2 0.50, Rf^3 0.70. *Anal.* Calcd for $C_{59}H_{84}N_{10}O_{15}S \cdot 2H_2O$: C, 57.1; H, 7.10; N, 11.3. Found: C, 57.1; H, 6.95; N, 11.4.

Boc–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl (5)——The title compound was obtained from Boc–Gln–ONp (0.12 g) and H–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl·TFA (prepared from 0.22 g of **4** and 0.75 ml of TFA containing 0.14 ml of 3,5-dimethylanisole and 0.02 ml of 1,2-ethanedithiol) and reprecipitated from DMF and AcOEt, yield 0.19 g (79.2%), mp 262—264 °C, $[\alpha]_D^{25}$ −49.0° (c=0.2, DMSO), Rf^2 0.54, Rf^3 0.68. *Anal.* Calcd for $C_{64}H_{92}N_{12}O_{17}S \cdot 2.5H_2O$: C, 55.8; H, 7.04; N, 12.3. Found: C, 55.8; H, 6.88; N, 12.7.

Boc–Ser–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl (6)——The title compound was obtained from Boc–Ser–N_3 (prepared from 0.08 g of Boc–Ser–N_2H_3 and 0.06 ml of isopentyl nitrite as usual) and H–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl·TFA (prepared from 0.18 g of **5** and 0.58 ml of TFA containing 0.1 ml of 3,5-dimethylanisole and 0.02 ml of 1,2-ethanedithiol) in the same way as described for the synthesis of Boc–Asn–Tyr–Lys(Z)–OBzl, yield 0.15 g (77.1%), mp 240 °C (dec.), $[\alpha]_D^{25}$ −43.1° (c=0.1, DMSO), Rf^3 0.78, Rf^4 0.62. *Anal.* Calcd for $C_{67}H_{97}N_{13}O_{19}S \cdot 3H_2O$: C, 54.6; H, 6.99; N, 12.4. Found: C, 54.4; H, 7.03; N, 12.6.

Z–Ser–Ala–His–Ser–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl (7)——The title compound was obtained from Z–Ser–Ala–His–N_3 (prepared from 0.85 g of **3** and 0.25 ml of isopentyl nitrite as usual) and H–Ser–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys(Z)–OBzl·TFA (prepared from 0.53 g of **6** and 1.6 ml of TFA containing 0.27 ml of 3,5-dimethylanisole and 0.1 ml of 1,2-ethanedithiol) in the same way as described for the synthesis of Boc–Asn–Tyr–Lys(Z)–OBzl, yield 0.34 g (52.5%), mp >300 °C, $[\alpha]_D^{25}$ −25.8° (c=0.1, DMF), Rf^5 0.45, Rf^6 0.75. *Anal.* Calcd for $C_{82}H_{112}N_{18}O_{23}S \cdot 7H_2O$: C, 52.5; H, 6.72; N, 13.4. Found: C, 52.5; H, 6.37; N, 13.4. Amino acid ratios in a 6 N HCl hydrolysate: Ser 1.94, Ala 1.94, His 0.75, Glu 1.15, Val 0.79, Gly 1.04, Met 0.82, Asp 0.99, Tyr 0.86, Lys 1.00 (average recovery 77.8%).

H–Ser–Ala–His–Ser–Gln–Val–Val–Ala–Gly–Met–Asn–Tyr–Lys–OH (I)——The protected tridecapeptide (350 mg) was treated with MSA–thioanisole (1.6 ml–1.2 ml) in the presence of *m*-cresol (1.1 ml) in an ice-bath for 30 min and at room temperature for 60 min, and dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h, dissolved in H_2O (10 ml) containing dithiothreitol (50 mg), treated with Amberlite IRA 45 (acetate form), for 30 min with stirring, and then filtered. The pH of the filtrate was adjusted to 8.0 with 1 N NH_4OH and readjusted to 6.5 with 1 N AcOH after 30 min of stirring in an ice-bath. The solution was lyophilized to give a hygroscopic powder, which was dissolved in H_2O (5 ml), and stirred with dithiothreitol (100 mg) at room temperature overnight. Then the solution was applied to a column of Sephadex G-25 (3.4 × 105 cm), which was eluted with 3% AcOH. The ultraviolet absorption at 280 nm was determined for each fraction (3 g). The fractions corresponding to the main peak (tube Nos. 85—104) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 120 mg. Subsequent purification was performed by reversed-phase HPLC on a YMC D-ODS-5 column (20 × 250 mm). A part of the above Sephadex G-25 treated sample (12 mg) was dissolved in 0.1% TFA aq. (1 ml) and the solution was applied to a column, which was eluted with a gradient of CH_3CN (15% to 30% in 30 min) in 0.1% TFA aq. at a flow rate of 15 ml per min. The effluent corresponding to the main peak (retention time 18 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 6.6 mg (55%), $[\alpha]_D^{25}$ −92.9° (c=0.07, H_2O), Rf^6 0.70; retention time 19 min in HPLC on an analytical YMC R-ODS-5 column (Waters, 4.6 × 250 mm) by gradient elution with 0.1% TFA in CH_3CN (15% to 30%, 30 min) in 0.1% TFA aq. at a flow rate of 1 ml per min (Fig. 2). Amino acid ratios in a 6 N HCl

hydrolysate: Ser 1.90, Ala 1.88, His 1.01, Glu 1.03, Val 0.71, Gly 0.96, Met 0.66, Asp 0.96, Tyr 0.81, Lys 1.00 (average recovery 74.4%).

Boc–Gln–Val–Val–Ala–Gly–OMe (II)——The title compound was obtained from Boc–Gln–ONp (1.2 g) and H–Val–Val–Ala–Gly–OMe·HCl (prepared from 1.6 g of Z–Val–Val–Ala–Gly–OMe[7] by catalytic hydrogenation) reprecipitated from DMF and MeOH, yield 1.3 g (65.8%), mp 246—248 °C, $[\alpha]_D^{22} -31.6°$ (c=0.9, DMSO), Rf^1 0.34, Rf^2 0.34. *Anal.* Calcd for $C_{26}H_{46}N_6O_9$: C, 53.3; H, 7.86; N, 14.1. Found: C, 53.1; H, 7.88; N, 14.3.

H–Gln–Val–Val–Ala–Gly–OMe·HCl (III)——II (1.0 g) was treated with 7 N HCl/dioxane (2.4 ml). After 10 min, dioxane (2.4 ml) was added to the solution. The reaction mixture was stirred at room temperature for 50 min. Ether was added to the solution to yield a white precipitate, which was collected by centrifugation and dried over KOH pellets *in vacuo*, yield 0.8 g (90.0%), mp 277—278 °C, $[\alpha]_D^{22} -54.8°$ (c=1.7, H_2O), Rf^5 0.46, Rf^6 0.72. *Anal.* Calcd for $C_{21}H_{38}N_6O_7$·HCl·H_2O: C, 46.7; H, 7.58; N, 15.1. Found: C, 47.0; H, 7.67; N, 14.9.

Acetyl-Gln–Val–Val–Ala–Gly–OMe (IV)——The title compound was prepared from AcONp[19] (0.13 g) and III (0.31 g), yield 0.18 g (56.8%), mp 295 °C (dec.), $[\alpha]_D^{24} -16.3°$ (c=0.8, DMSO), Rf^1 0.24, Rf^2 0.35, Rf^3 0.79. *Anal.* Calcd for $C_{23}H_{40}N_6O_8$·1/2H_2O: C, 51.4; H, 7.63; N, 15.6. Found: C, 51.7; H, 7.55; N, 15.7.

Benzoyl-Gln–Val–Val–Ala–Gly–OMe (V)——The title compound was prepared from benzoyl chloride (0.1 ml) and III (0.31 g), yield 80 mg (22.9%), mp 298 °C (dec.), $[\alpha]_D^{22} -25.9°$ (c=0.5, DMSO), Rf^1 0.26, Rf^2 0.38, Rf^3 0.79. *Anal.* Calcd for $C_{28}H_{42}N_6O_8$·3/4H_2O: C, 55.7; H, 7.21; N, 13.9. Found: C, 55.7; H, 7.14; N, 14.2.

β-Naphthoyl-Gln–Val–Val–Ala–Gly–OMe (VI)——The title compound was prepared from β-naphthoyl chloride (0.14 g) and III (0.31 g), yield 0.22 g (57.2%), mp >300 °C, $[\alpha]_D^{22} -4.4°$ (c=0.6, DMSO), Rf^1 0.26, Rf^2 0.50, Rf^3 0.79. *Anal.* Calcd for $C_{32}H_{44}N_6O_8$·1/4H_2O: C, 59.6; H, 6.90; N, 13.0. Found: C, 59.6; H, 6.87; N, 13.3.

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Studies on Peptides. CLIII.[1,2] Application of the Hard-Acid Deprotecting Procedure to the Synthesis of Ovine Corticotropin Releasing Factor (oCRF)

KENICHI AKAJI,[a] MOTOYOSHI NOMIZU,[a] KAZUHIDE WATANABE,[a] SUSUMU FUNAKOSHI,[a] HIROO IMURA,[b] TOSHIHIKO TSUKADA,[b] JUNICHI FUKATA,[b] HIROMICHI NAKAMUTA,[c] MASAO KOIDA,[c] NOBUTAKA FUJII,[a] and HARUAKI YAJIMA*,[a]

Faculty of Pharmaceutical Sciences,[a] The 2nd Internal Medicine,[b] Kyoto University, Sakyo-ku, Kyoto 606 Japan, and Department of Faculty of Pharmaceutical Sciences, Setsunan University,[c] Hirakata, Osaka 573-01, Japan

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A 41-residue peptide corresponding to the entire amino acid sequence of ovine corticotropin releasing factor (oCRF) was synthesized by assembling ten peptide fragments of established purity, followed by hard-acid deprotection with 1 M trimethylsilyl trifluoromethanesulfonate in trifluoroacetic acid. β-Cycloheptylaspartate was employed to minimize base-catalyzed succinimide formation. When tested by *in vivo* assay, synthetic oCRF was as active as synthetic hCRF in terms of ability to release immunoreactive corticotropin.

Keywords——ovine corticotropin releasing factor synthesis; β-cycloheptylaspartate; thioanisole-mediated deprotection; hard-acid deprotection; trimethylsilyl trifluoromethanesulfonate deprotection; base-catalyzed succinimide formation; immunoreactive corticotropin release

Following the synthesis of human corticotropin releasing factor (hCRF),[3] we wish to present a detailed account of our solution phase synthesis of ovine CRF (oCRF), the structure of which was elucidated by Vale *et al.*[4] in 1981. Several research groups have reported solid-phase[4,5] or, preliminarily, conventional solution-phase syntheses[6] of this hypothalamic peptide. However, no detailed information on the solution-phase method is available at the present time. From a synthetic viewpoint, especially in the solution-phase synthesis, CRF offers particular difficulty in the elongation of the peptide chain, presumably due to its high helical content.[7]

Different from our previous synthesis of hCRF,[3] our newly developed hard-acid deprotecting procedure[8] was employed in the present oCRF synthesis. The TFA-labile Z(OMe)[9] or the Boc group was employed for N^{α}-protection. In the final step of the synthesis, all side-chain protecting groups were cleaved by treatment with 1 M TMSOTf–thioanisole/TFA, *i.e.*, Z from Lys, Bzl from Glu, Asp and Ser, Mts[10] from Arg, and Chp from Asp (position 25). Asp(OChp)[11] was employed for preparation of the Asp–Gln sequence in order to minimize base-catalyzed succinimide formation.[12] Ten peptide fragments were selected as building blocks to construct the entire peptide backbone of oCRF. This factor possesses the same sequence as hCRF,[13] except for replacement of seven residues at positions 2, 22, 23, 25, 38, 39, and 41, *i.e.*, Glu, Ala, Arg, Glu, Met, Glu, and Ile of hCRF with Gln, Thr, Lys, Asp, Leu, Asp, and Ala, respectively. Thus, of the ten fragments, six, [2], [3], [4], [7], [8], and [9], are identical with those employed for the hCRF synthesis and four, [1], [5], [6], and [10], which cover the area of species variation, were newly synthesized.

The C-terminal fragment, Boc–Leu–Asp(OBzl)–Ile–Ala–NH_2 [1], was prepared in a

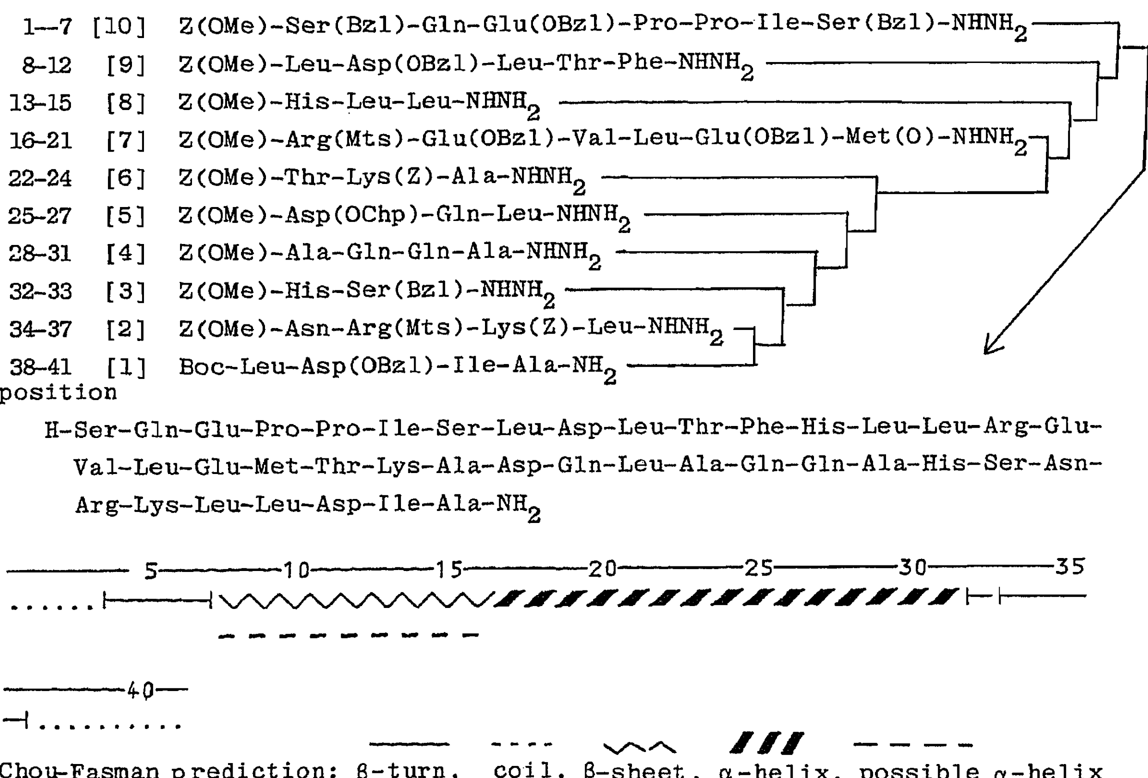

Fig. 1. Synthetic Route to oCRF and Prediction of Its Conformation

stepwise manner starting from H–Ala–NH_2, onto which the respective three amino acid residues were condensed by the mixed anhydride (MA),[14] the Np[15] and again the MA procedures, respectively. Fragment [5] contains the Asp–Gln sequence, which is somewhat susceptible to base-catalyzed succinimide formation.[12] In order to minimize this side reaction, Z(OMe)–Asp(OChp)–OH, instead of Z(OMe)–Asp(OBzl)–OH, was condensed with a TFA-treated sample of Z(OMe)–Gln–Leu–NHNH–Troc[16] by the Su procedure[17] and from the resulting tripeptide derivative, the Troc group[18] was removed by treatment with Cd/AcOH[19] to afford [5]. Fragment [6], Z(OMe)–Thr–Lys(Z)–Ala–$NHNH_2$, was easily prepared by condensation of Z(OMe)–Thr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Lys(Z)–Ala–OMe[20] *via* the azide,[21] followed by the usual hydrazine treatment. Fragment [10], Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$, was prepared by stepwise condensations of Z(OMe)–Gln–OH and Z(OMe)–Ser(Bzl)–OH onto a TFA-treated sample of Z(OMe)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc[3] *via* the Np ester and then the MA procedures, respectively, followed by the usual Zn/AcOH treatment.[22]

Ten fragments were then assembled successively, as shown in Fig. 1, by the azide procedure to minimize racemization. As a solvent, DMF or DMF–DMSO (1 : 1) was employed and the amount of the acyl component was increased from 1.1 to 7 eq as chain elongation progressed. Condensations of fragments from [1] to [7] proceeded as usual without particular difficulty. However, in the subsequent azide condensations of fragments from [8] to [10], each acyl component had to be used in a large excess (7 eq) in order to bring the reaction to completion and every reaction had to be performed at a lower temperature (−18 °C) than usual (+4 °C) in order to minimize Curtius rearrangement.[23] As reported in our previous hCRF synthesis, helical or β-sheet conformation of CRF may be responsible for such unusual phenomena in the chain elongation reactions. Each protected product was purified either by precipitation from DMF with MeOH or by gel-filtration on Sephadex LH-60 using DMF as

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic oCRF and Its Intermediates

	Protected peptides									Syn. oCRF	Residue
	34—41	32—41	28—41	25—41	22—41	16—41	13—41	8—41	1—41		
Asp	2.01	2.05	2.09	3.00	3.04	3.23	3.15	4.40	4.27	3.85	(4)
Thr					0.86	0.91	0.94	2.01	2.29	1.99	(2)
Ser		0.95	0.90	1.01	1.00	1.03	1.10	1.18	3.34	3.03	(3)
Glu			2.08	2.80	3.01	5.23	4.88	5.06	7.25	7.29	(7)
Pro									1.75	1.70	(2)
Ala	1.07	1.05	3.32	2.96	4.04	4.41	4.12	4.19	4.32	4.09	(4)
Val						0.73	0.81	0.64	1.12	1.02	(1)
Met						0.68	0.79	0.67	0.83	1.06	(1)[a]
Ile	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.00	2.00	(2)
Leu	2.02	2.01	2.05	2.81	3.03	3.95	6.07	8.27	7.60	7.58	(8)
Phe								0.87	1.00	0.95	(1)
Lys	1.01	1.06	0.95	1.06	1.86	2.02	1.97	1.99	2.12	2.00	(2)
His		1.06	0.98	0.95	1.00	1.07	1.99	1.85	1.69	1.89	(2)
Arg	1.04	0.99	0.89	0.98	0.96	2.05	1.87	1.82	1.91	1.81	(2)
Rec. %	76	78	82	83	83	73	84	70	71	80	

a) Met+Met(O).

an eluant. Throughout this synthesis, Ile was used as a diagnostic amino acid (Table I). After each condensation, each product was subjected to 6 N HCl hydrolysis and the recovery of Ile was compared with those of newly added amino acids to assure satisfactory incorporation. The homogeneity of each product was further ascertained by elemental analysis and thin layer chromatography (TLC).

In the final step, protected oCRF was treated with 1 M TMSOTf–thioanisole/TFA in the presence of *m*-cresol to remove all protecting groups employed, except Met(O), which is known to be partially reduced under this deprotecting condition.[16] The deprotected peptide was purified in essentially the same manner as described in the final step of our hCRF synthesis: 1) Treatment with dil. ammonia containing NH_4F to reverse any possible N→O shift[24] and ensure the complete hydrolysis of trimethylsilyl residues. 2) Incubation with dithiothreitol to ensure the complete reduction of Met(O). 3) Gel-filtration on Sephadex G-50. 4) Ion-exchange chromatography on CM-cellulose. 5) Desalting on Diaion HP-20. 6) High performance liquid chromatography (HPLC) on Vydac 5C18. The peptide thus purified exhibited a sharp single spot on TLC and a single band in disk isoelectrofocusing (Pharmalyte, pH 3—10). Its purity was further confirmed by acid hydrolysis and leucine aminopeptidase (LAP) digestion.

When tested in rats anesthetized with sodium pentobarbital (50 mg/kg) according to Rivier *et al.*,[25] our synthetic oCRF (5.0 μg/kg) significantly elevated the plasma immunoreactive corticotropin (ACTH) level (76% ± 23) and its potency was judged to be equivalent to that of synthetic hCRF.

Experimental

General experimental methods employed here are essentially the same as described in our hCRF synthesis.[3]

Unless otherwise stated, products were purified by one of the following procedures. Procedure A: For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized or precipitated from appropriate solvents. Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O and

crystallized or precipitated from appropriate solvents.

HPLC was conducted with a Waters 204 compact model. TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v). Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 $CHCl_3$–MeOH (10 : 0.5), Rf_3 $CHCl_3$–MeOH (9 : 1), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24).

An ACTH immunoassay kit was purchased from Japan Radioisotope Association (Bunkyo-ku, Tokyo).

Z(OMe)–Ile–Ala–NH_2——The title compound was prepared by the MA procedure and purified by procedure B followed by recrystallization from DMF with MeOH; yield 62%, mp 229—231 °C, $[\alpha]_D^{20}$ +3.9° (c = 1.0, DMSO), Rf_1; 0.75. *Anal.* Calcd for $C_{18}H_{27}N_3O_5$: C, 59.15; H, 7.45; N, 11.50. Found: C, 58.86; H, 7.48; N, 11.33.

Boc–Asp(OBzl)–Ile–Ala–NH_2——A mixture of Boc–Asp(OBzl)–ONp (20.1 g, 45.2 mmol) and a TFA-treated sample of the above dipeptide (15.0 g, 41.0 mmol) in DMF (150 ml) containing Et_3N (11.5 ml, 82.1 mmol) was stirred at 4 °C for 12 h, then concentrated. The residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 16.6 g (80%), mp 198—200 °C, $[\alpha]_D^{20}$ −26.1° (c = 1.1, DMSO), Rf_1 0.81. *Anal.* Calcd for $C_{25}H_{38}N_4O_7$: C, 59.27; H, 7.56; N, 11.06. Found: C, 59.00; H, 7.50; N, 10.98.

Boc–Leu–Asp(OBzl)–Ile–Ala–NH_2 [1]——An MA [prepared from 9.6 g (41.5 mmol) of Boc–Leu–OH] in THF was added to an ice-chilled solution of a TFA-treated sample of the above tripeptide amide (14.0 g, 27.6 mmol) in DMF (100 ml) containing Et_3N (3.8 ml, 27.6 mmol) and the mixture was stirred at 4 °C for 5 h. The product was purified by procedure B followed by precipitation from DMF with AcOEt; yield 10.33 g (60%), mp 222—225 °C, $[\alpha]_D^{20}$ −32.3° (c = 1.1, DMSO), Rf_1 0.83. Amino acid ratios in 6 N HCl hydrolysate: Leu 1.10, Asp 1.04, Ile 1.00, Ala 1.00 (recovery of Ala, 69%). *Anal.* Calcd for $C_{31}H_{49}N_5O_8 \cdot 1/2H_2O$: C, 59.21; H, 8.01; N, 11.14. Found: C, 59.24; H, 7.96; N, 11.19.

Z(OMe)–Asp(OChp)–Gln–Leu–NHNH–Troc——A mixture of Z(OMe)–Asp(OChp)–OSu (4.80 g, 9.79 mmol), Et_3N (2.5 ml, 17.9 mmol) and a TFA-treated sample of Z(OMe)–Gln–Leu–NHNH–Troc[16] (5.0 g, 8.16 mmol) in DMF (50 ml) was stirred at room temperature for 12 h. The product was purified by procedure A followed by recrystallization from AcOEt and ether; yield 4.85 g (72%), mp 164—166 °C, $[\alpha]_D^{20}$ −15.8° (c = 0.8, DMF), Rf_1 0.74. *Anal.* Calcd for $C_{34}H_{49}Cl_3N_6O_{11}$: C, 49.55; H, 5.99; N, 10.20. Found: C, 49.25; H, 6.02; N, 10.23.

Z(OMe)–Asp(OChp)–Gln–Leu–$NHNH_2$——The above tripeptide derivative (3.50 g, 4.25 mmol) in MeOH–AcOH (20 ml—5 ml) was treated with Cd powder (4.8 g, 10 eq) at room temperature for 12 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 5% EDTA. The resulting powder was washed with 5% EDTA and H_2O and then precipitated from MeOH with ether; yield 2.02 g (73%); mp 191—193 °C, $[\alpha]_D^{15}$ −11.7° (c = 1.0, DMF), Rf_1 0.62. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Glu 0.99, Leu 1.00 (recovery of Leu, 86%). *Anal.* Calcd for $C_{31}H_{48}N_6O_9$: C, 57.39; H, 7.46; N, 12.96. Found: C, 57.27; H, 7.61; N, 12.85.

Z(OMe)–Thr–Lys(Z)–Ala–OMe——The azide [prepared from 3.37 g (11.3 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (10 ml) and Et_3N (2.9 ml, 20.8 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Lys(Z)–Ala–OMe[20] (5.00 g, 9.44 mmol) in DMF (50 ml) and the mixture was stirred at 4 °C for 12 h. The product was purified by procedure B followed by precipitation from DMF with MeOH; yield 4.70 g (79%), mp 153—156 °C, $[\alpha]_D^{20}$ −1.0° (c = 1.0, DMF), Rf_2 0.40. *Anal.* Calcd for $C_{31}H_{42}N_4O_{10}$: C, 59.03; H, 6.71; N, 8.89. Found: C, 58.89; H, 6.74; N, 8.80.

Z(OMe)–Thr–Lys(Z)–Ala–$NHNH_2$ [6]——The above tripeptide ester (2.50 g, 3.96 mmol) in DMF (30 ml) was treated with 80% hydrazine hydrate (2.4 ml, 10 eq) at room temperature for 12 h, then the solution was concentrated and the residue was precipitated from DMSO with MeOH; yield 2.46 g (98%), mp 214—217 °C, $[\alpha]_D^{20}$ +3.9° (c = 1.0, DMSO), Rf_1 0.49. Amino acid ratios in 6 N HCl hydrolysate: Thr 1.02, Lys 1.00, Ala 1.12 (recovery of Lys, 88%). *Anal.* Calcd for $C_{30}H_{42}N_6O_9$: C, 57.13; H, 6.71; N, 13.33. Found: C, 56.96; H, 6.72; N, 13.43.

Z(OMe)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A mixture of Z(OMe)–Gln–ONp (1.19 g, 2.76 mmol), Et_3N (0.74 ml, 5.27 mmol) and a TFA-treated sample of Z(OMe)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc[3] (2.70 g, 2.51 mmol) in DMF (20 ml) was stirred at 4 °C for 24 h. The product was purified by procedure A followed by column chromatography on silica gel (4.3 × 9 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was recrystallized from MeOH and isopropyl ether; yield 1.91 g (63%), mp 98—100 °C, $[\alpha]_D^{15}$ −23.8° (c = 1.0, DMF), Rf_3 0.42. *Anal.* Calcd for $C_{55}H_{70}Cl_3N_9O_{15}$: C, 54.88; H, 5.86; N, 10.47. Found: C, 54.87; H, 5.93; N, 10.46.

Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——An MA [prepared from 0.68 g (1.90 mmol) of Z(OMe)–Ser(Bzl)–OH] in THF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of the above protected hexapeptide (1.90 g, 1.58 mmol) in DMF (15 ml) containing Et_3N (0.22 ml, 1.58 mmol) and the mixture was stirred at 4 °C for 4 h. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 1.72 g (79%), mp 89—91 °C, $[\alpha]_D^{15}$ −22.9° (c = 1.0, DMF), Rf_3 0.40. *Anal.* Calcd for $C_{65}H_{81}Cl_3N_{10}O_{17}$: C, 56.54; H, 5.91; N, 10.15. Found: C, 56.51; H, 5.78; N, 10.05.

Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$ [10]——The above protected heptapeptide derivative (1.40 g, 1.01 mmol) in MeOH–AcOH (5 ml–5 ml) was treated with Zn powder (0.66 g, 10 eq) at room temperature for 18 h. The mixture was filtered, the filtrate was concentrated and the residue was dissolved in *n*-BuOH. The organic phase was washed with 5% EDTA and H_2O and then concentrated. The residue was treated with

TABLE II. Characterization of Protected oCRF and Its Intermediates

	Puri. proc. Yield (%)	Rf_1	mp (°C)	$[\alpha]_D^{15}$ (DMF)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Z(OMe)-(34—41)-NH_2	C 83	0.74	253—256	−26.6°	$C_{74}H_{106}N_{14}O_{18}S$	58.79 (58.50	7.07 7.09	12.97 13.06)
Z(OMe)-(32—41)-NH_2	C 93	0.63	232—234	−19.2°	$C_{90}H_{124}N_{18}O_{21}S \cdot 4H_2O$	56.94 (56.93	7.01 6.98	13.28 13.17)
Z(OMe)-(28—41)-NH_2	C 76	0.25	214—216	−16.9°	$C_{106}H_{150}N_{24}O_{27}S$	57.23 (56.95	6.80 6.93	15.11 15.32)
Z(OMe)-(25—41)-NH_2	C 69	0.57	250—252	−48.5°	$C_{128}H_{186}N_{28}O_{33}S \cdot 6H_2O$	55.20 (55.13	7.17 6.92	14.08 13.86)
Z(OMe)-(22—41)-NH_2	C 82	0.46	263—264	−47.5°	$C_{149}H_{216}N_{32}O_{39}S \cdot 7H_2O$	55.27 (55.09	7.16 6.87	13.85 13.68)
Z(OMe)-(16—41)-NH_2	D 43	0.64	238—239	−15.2°	$C_{204}H_{293}N_{41}O_{52}S_3 \cdot 8H_2O$	55.78 (56.00	7.09 6.83	13.08 12.80)
Z(OMe)-(13—41)-NH_2	D 73	0.60	230—231	−8.2°	$C_{222}H_{322}N_{46}O_{55}S_3 \cdot 3H_2O$	57.15 (57.07	7.09 7.12	13.81 13.92)
Z(OMe)-(8—41)-NH_2	D 78	0.58	226—228	−5.9°	$C_{258}H_{371}N_{51}O_{63}S_3 \cdot 7H_2O$	57.20 (57.25	7.16 7.08	13.19 13.28)
Z(OMe)-(1—41)-NH_2	D 66	0.65	185—186	−7.8°	$C_{311}H_{439}N_{59}O_{75}S_3 \cdot 4H_2O$	58.61 (58.53	7.07 7.22	12.97 12.93)

Purification procedure: C, precipitation from DMF with MeOH; D, gel-filtration on Sephadex LH-60.

isopropyl ether and the resulting powder was precipitated from MeOH with ether; yield 0.76 g (62%), mp 93—95 °C, $[\alpha]_D^{15}$ −24.2° (c=1.0, DMF), Rf_1 0.46. Amino acid ratios in 6 N HCl hydrolysate: Ser 2.00, Glu 2.05, Pro 2.22, Ile 1.00 (recovery of Ile, 97%). *Anal.* Calcd for $C_{62}H_{80}N_{10}O_{15} \cdot H_2O$: C, 60.87; H, 6.76; N, 11.45. Found: C, 60.73; H, 6.59; N, 11.19.

Synthesis of Protected oCRF——Successive azide condensations of the ten fragments were carried out according to the indicated route (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 1 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF or DMF–DMSO (1 : 1, for condensations of [2], [3], [4], and [6]) containing Et_3N (1 eq). The corresponding azide (the amount was increased from 1.1 to 7 eq as chain elongation progressed) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at 4 °C (condensations from [1] to [7]) or −18 °C (condensations from [8] to [10]) as described in the text, until the solution became negative to the ninhydrin test. H_2O was added and the resulting powder was purified by precipitation from DMF with MeOH (procedure C) or by gel-filtration on Sephadex LH-60 using DMF as an eluant (procedure D). In the latter case, eluates (10 ml each) were examined by measuring the ultraviolet absorption (UV) at 280 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to form a powder. Purification procedures, yields, physical constants and analytical data of protected oCRF and its protected intermediates are listed in Table II.

H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2 (oCRF)——Protected oCRF (55 mg, 8.7 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (3.9 ml) in the presence of m-cresol (128 μl, 140 eq) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O–MeOH (5 ml–1 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH containing NH_4F (35 eq) and after 30 min, to 6.0 with 1 N AcOH. Dithiothreitol (135 mg, 100 eq) was added and the solution, after being incubated at 37 °C for 12 h, was applied to a column of Sephadex G-50 (2.4 × 133 cm), which was eluted with 1 N AcOH. Individual fractions (5.5 ml) was examined by the Folin–Lowry test[26)] (optical density at 750 nm). The fractions corresponding to the front main peak (tube Nos. 34—

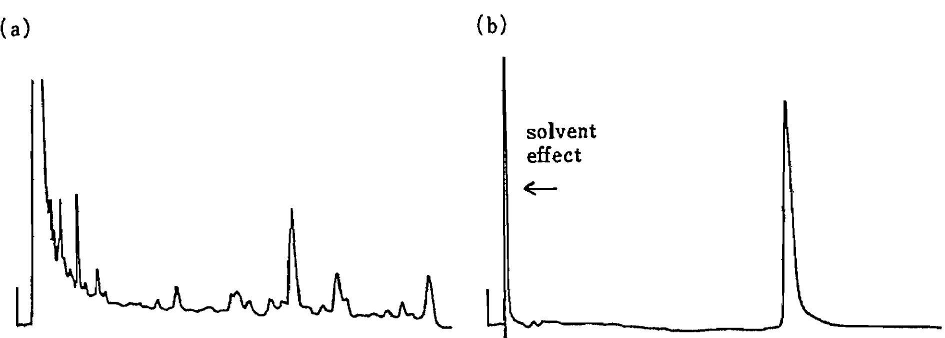

Fig. 2. HPLC of Synthetic oCRF (OD at 233 nm)
(a) CM-Purified sample. (b) Purified synthetic oCRF.

47) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 37 mg (91%).

The crude product thus obtained (15 mg) was dissolved in pH 5.0, 0.02 M $AcONH_4$ buffer (5 ml) containing 3 M urea and the solution was applied to a column of CM-cellulose (1.6 × 4 cm), which was eluted with pH 5.0, 0.2 M $AcONH_4$ (200 ml) containing 3 M urea through a mixing flask containing the starting buffer (100 ml). The fractions (4 ml each) corresponding to the main peak (tube Nos. 23—27, monitored by the Folin–Lowry test) were combined and the solvent was removed by lyophilization. The residue was dissolved in 5% acetonitrile in 1 N AcOH and the solution was applied to a column of Diaion HP-20 (1.6 × 5 cm), which was first washed with the same solvent (120 ml) to remove urea and the salt and then eluted with a gradient of acetonitrile (5 to 50%) in 1 N AcOH (300 ml). The fractions (4 ml each) corresponding to the main peak (tube Nos. 35—60, monitored by the Folin–Lowry test) were combined and the solvent was removed by lyophilization to give a white fluffy powder. The rest of the crude sample was similarly purified; total yield 15.0 mg (37%).

Subsequent purification was performed by reverse-phase HPLC on a Vydac 5C18 column (4.6 × 250 mm). The above CM-purified sample was dissolved in 0.5 N AcOH (0.7 ml) containing 5% 2-mercaptoethanol and the solution was incubated at 37 °C for 12 h in order to reduce the Met(O) residue formed during manipulation. The solution (100 μl each) was applied to the HPLC column, which was eluted with a gradient of acetonitrile (32 to 37% in 60 min) in 0.1% TFA at a flow rate of 0.8 ml/min. The eluate corresponding to the main peak (Fig. 2a; retention time, 45.3 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 0.3 mg. The rest of the sample was similarly purified; total yield, 4.1 mg; overall yield from protected oCRF, 10.1%. $[\alpha]_D^{15}$ −136.4° (c=0.03, 1 N AcOH); Rf_4 0.43, Rf_5 0.36; retention time 45.3 min in HPLC on a Vydac 5C18 column (4.6 × 250 mm) by gradient elution as stated above (Fig. 2b). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in an LAP digest (numbers in parentheses are theoretical): Asp 3.23 (3), Thr 1.78 (2), Ser 1.86 (2), Glu 2.41 (3), Pro 0.75 (2), Ala 3.80 (4), Val 1.09 (1), Met 0.81 (1), Ile 1.58 (2), Leu 7.47 (8), Phe 1.10 (1), Lys 2.00 (2), His 1.67 (2), Arg 1.95 (2), Asn (1) and Gln (4) were not determined (recovery of Lys 70%, incomplete digestion of the Glu–Pro–Pro–Ile sequence was presumably due to poor prolidase activity of this enzyme preparation).

References and Notes

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used. Bzl = benzyl, Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylenesulfonyl, Chp = cycloheptyl, Troc = 2,2,2-trichloroethyloxycarbonyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, THF = tetrahydrofuran, DMF = dimethylformamide, DMSO = dimethylsulfoxide, EDTA = ethylenediaminetetraacetic acid disodium salt, CM = carboxymethyl.

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Conformational Analysis of Curdione and Neocurdione

KENZO HARIMAYA,[a] TAMIKO OHKURA,[a] JI-FU GAO,[1)] YOICHI IITAKA,[b] EIJI ŌSAWA,[c] and SEIICHI INAYAMA*,[a]

Pharmaceutical Institute, School of Medicine, Keio University,[a] 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan, Faculty of Pharmaceutical Sciences, The University of Tokyo,[b] Hongo, Bunkyo-ku, Tokyo 113, Japan and Department of Chemistry, Faculty of Science, Hokkaido University,[c] Sapporo 060, Japan

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Molecular mechanics calculations of flexible germacrenediones, especially curdione (**1**) and neocurdione (**2**), isolated from *Curcuma wenyujin* (Zingiberaceae), were used to identify the stable conformations among forty-eight conformers for each compound. The calculated ratios (70 : 30 for **1** and 68 : 32 for **2**) of conformations of *anti* and *syn* type ($C(5)=O/C(10)-CH_3$) are in reasonably good accordance with the ratios (5 : 1 for **1** and 2 : 1 for **2**) which were estimated from low-temperature nuclear magnetic resonance (NMR) measurements. The populations of the intermediate conformations considered to be involved in the biomimetic conversions from **1** to curcumol (**3**) and curcumalactone (**4**) were found to be 11.9% of *syn* type and 1.0% of *anti* type, respectively.

Keywords——curdione; neocurdione; germacrenedione; *Curcuma wenyujin*; molecular mechanics calculation; MM2; conformational analysis

Curdione[2,3)] and neocurdione[4,5)] isolated from *Curcuma wenyujin* (Zingiberaceae) have been shown to have the absolute configurations **1** and **2**, respectively. In our preceding papers, the possible existence of *anti* and *syn* conformations of both **1**[2)] and **2**[5)] was deduced from the appearance of two signals at 5.06 and 5.63 ppm in **1** and at 4.98 and 5.48 ppm in **2**. These were attributed to the C(1) olefinic proton under the influence of the anisotropic effect of the C(5) carbonyl in low-temperature nuclear magnetic resonance (NMR) studies. Furthermore, the X-ray diffraction analysis of **1**[2,3)] has shown a usual chair/chair conformation, in which the methyl group at C(10) and the carbonyl oxygen on C(5) are oriented in opposite directions with respect to the mean plane of the ten-membered ring system (*anti*). On the other hand, the X-ray crystallographic study of **2** (*i.e.*, 7-epicurdione)[4)] has revealed an unusual chair/twist-boat conformation, wherein the C(10) and the C(5) substituents are on the same side of the average molecular plane (*syn*). This sharp contrast in the conformations of **1** and **2** in the solid state and the similar observation of the existence of both *anti* and *syn* conformations in solution for **1** and **2** prompted us to apply molecular mechanics calculations to these flexible germacrenedione ring systems. Furthermore, since biomimetic transformations from **1** to

1
2 : 7αH

3

4

Chart 1

curcumol (3)[6] and curcumalactone (4)[3] have been achieved, it is of much interest to clarify the stabilities of the intermediate conformations of **1** in the processes of formation of **3** and **4**.

Methods

The X-ray crystallographic and NMR observations are interpreted by the molecular mechanics calculations as follows. Manipulation of molecular models of **1** or **2** indicates that the dihedral angle at C(4)–C(5)–C(6)–C(7) is 180° (±20°), as is that at C(9)–C(10)–C(1)–C(2). It is also clear that the C(10) and C(5) substituents are approximately perpendicular to the average plane of the molecule. The four principal conformations thus deduced are designated as *anti*-up (I), *syn*-up (II), *anti*-down (III) and *syn*-down (IV), where the terms up and down refer to the orientation of the C(5)-carbonyl group relative to the average molecular plane (Tables I and II). Molecular models further revealed that both the C(8)-carbonyl and the C(3)-methylene of **1** and **2** could readily flip. The flipping leads to two discrete rotamers around the C(2)–C(3) bond and also two around the C(7)–C(8) bond. This gives rise to four sub-conformations for each of the principal conformations, I to IV, and hence to sixteen conformations (**1** to **16**) of each compound (Tables I and II). Finally, three staggered rotamers (g^+, g^- and *anti*) of the C(7)–C(11) bond of the isopropyl side chain were taken into account for each of the conformations mentioned above. Thus, forty-eight

TABLE I. Relative Strain Energies (kcal/mol) and Populations (%) of Conformers of Curdione (**1**) Obtained by MM2 Calculation

Conformer (8—7—11—12)		Rel. S.E.	Pop.	Conformer (8—7—11—12)		Rel. S.E.	Pop.
I *anti*-up				III *anti*-down			
1	g^+	0.2448	10.08	9	g^+	3.2893	0.06
	g^-	0	15.24		g^-	3.1701	0.07
	anti	0.6449	5.13		*anti*	1.7702	0.77
			(30.45)				(0.90)
2	g^+	0.4961	6.60	10	g^+	5.0368	0
	g^-	0.0711	13.52		g^-	4.8922	0
	anti	0.3331	8.69		*anti*	3.2112	0.07
			(28.81)				(0.07)
3	g^+	2.7084	0.16	11	g^+	5.1596	0
	g^-	2.9084	0.11		g^-	5.9565	0
	anti	1.1642	2.14		*anti*	1.4913	1.23
			(2.41)				(1.23)
4	g^+	2.1344	0.42	12	g^+	7.0855	0
	g^-	2.4001	0.26		g^-	7.9206	0
	anti	0.5786	5.74		*anti*	2.8778	0.12
			(6.42)				(0.12)
II *syn*-up				IV *syn*-down			
5	g^+	1.5574	1.10	13	g^+	2.7809	0.14
	g^-	1.5764	1.09		g^-	2.3734	0.28
	anti	1.4715	1.27		*anti*	2.4758	0.23
			(3.46)				(0.65)
6	g^+	0.7553	4.25	14	g^+	4.0961	0.01
	g^-	0.6493	5.09		g^-	3.7058	0.03
	anti	1.0232	2.71		*anti*	3.3034	0.06
			(12.05)				(0.10)
7	g^+	1.6489	0.94	15	g^+	7.2391	0
	g^-	2.1080	0.43		g^-	8.1586	0
	anti	0.8500	3.63		*anti*	1.3991	1.44
			(5.00)				(1.44)
8	g^+	1.7264	0.83	16	g^+	9.7085	0
	g^-	2.3053	0.31		g^-	10.5686	0
	anti	0.5820	5.71		*anti*	3.5554	0.04
			(6.85)				(0.04)

TABLE II. Relative Strain Energies (kcal/mol) and Populations (%) of Conformers of Neocurdione (2) Obtained by MM2 Calculation

Conformer (8—7—11—12)		Rel. S.E.	Pop.	Conformer (8—7—11—12)		Rel. S.E.	Pop.
I *anti*-up				III *anti*-down			
1	g^+	5.4964	0	9	g^+	1.9377	0.66
	g^-	0.5329	7.07		g^-	0.4594	8.01
	anti	5.8878	0		*anti*	2.1398	0.47
			(7.07)				(9.14)
2	g^+	4.4917	0.01	10	g^+	3.4854	0.05
	g^-	1.0362	3.02		g^-	2.0045	0.59
	anti	5.3537	0		*anti*	3.6593	0.03
			(3.03)				(0.67)
3	g^+	3.6693	0.03	11	g^+	0.4510	8.13
	g^-	1.8163	0.81		g^-	0	17.39
	anti	3.1998	0.08		*anti*	0.0255	16.66
			(0.92)				(42.18)
4	g^+	2.8781	0.13	12	g^+	1.8348	0.79
	g^-	1.4598	1.48		g^-	1.8287	0.79
	anti	2.7906	0.15		*anti*	1.5262	1.32
			(1.76)				(2.90)
II *syn*-up				IV *syn*-down			
5	g^+	7.9458	0	13	g^+	1.6494	1.07
	g^-	1.8060	0.82		g^-	0.5906	6.42
	anti	8.7258	0		*anti*	2.1946	0.42
			(0.82)				(7.91)
6	g^+	6.5957	0	14	g^+	2.4672	0.27
	g^-	1.0063	3.18		g^-	1.6383	1.20
	anti	7.5047	0		*anti*	2.9709	0.11
			(3.18)				(1.58)
7	g^+	2.5691	0.22	15	g^+	0.6952	5.38
	g^-	1.9274	0.67		g^-	0.9150	3.71
	anti	2.1533	0.46		*anti*	0.6090	6.22
			(1.35)				(15.31)
8	g^+	2.2955	0.36	16	g^+	2.6185	0.21
	g^-	2.0722	0.52		g^-	2.5898	0.22
	anti	1.9396	0.66		*anti*	2.6131	0.21
			(1.54)				(0.64)

conformations for **1** and the same number of conformations for **2** were subjected to MM2 calculations with complete structure optimization.[7] All of these gave minimum energy structures.

Results and Discussion

The resulting relative strain energies and populations are summarized in Tables I and II. The most stable calculated conformation of **1** (conformer 1, 30.5% population) is coincident with that of its crystal structure.[2] The calculated ratio (70 : 30, 25 °C in gas phase) between *anti* (I+III) and *syn* (II+IV) conformers compares reasonably well with the ratio (5 : 1, −70 °C in solution) deduced from the aforementioned ^{1}H-NMR studies. In the case of **2**, the most stable conformation (conformer 11), amounting to 42.2% of the population, is different from the crystal conformer,[5] whose equivalent conformer 14 is calculated to amount to only less than 2% (25 °C in the gas phase). Nevertheless, the calculated ratio (68 : 32) between *anti* and *syn* conformers of **2** accords well with the observed one (2 : 1, −60 °C in solution) obtained from the NMR measurements. The populations of the intermediate

conformations considered to be involved in the course of the biomimetic transformation from **1** to **3** and **4** were found to amount to 11.9% (conformers 7 and 8) and 1.0% (conformers 9 and 10), respectively. The stabilities of intermediate conformations postulated to be involved in the conversions of **2** to the 7-epimers of **3** and **4** can similarly be evaluated from the MM2 data for **2** in Table II.

Experimental

Molecular mechanics calculations were carried out with the MM2 (molecular mechanics version 2), program written by one of the authors (E. O.) for a library program (TLIBY Y4MM2), using a HITAC M-280H computer at the computer centre, the University of Tokyo (see *Center News*, **17**, supplement 1, 94, 1985).

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Studies on the Constituents of *Actinostemma lobatum* MAXIM. II.[1] Structures of Actinostemmosides G and H, New Dammarane Triterpene Glycosides Isolated from the Herb

TOSHIHIRO FUJIOKA, YUKIKO IWASE, HIKARU OKABE,* KUNIHIDE MIHASHI and TATSUO YAMAUCHI

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma 8-19-1, Jonan-ku, Fukuoka 814-01, Japan

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From the less polar saponin fraction of the dried herb of *Actinostemma lobatum* MAXIM. (Cucurbitaceae), two new dammarane-type triterpene glycosides, actinostemmosides G and H, were isolated. The structures of actinostemmosides G and H were elucidated mainly on the basis of spectral evidence as (20*S*)-3*β*,6*α*,7*β*,20,27-ptentahydroxy-dammar-24-ene 20-*O*-*β*-D-glucopyranoside and (20*S*)-3*β*,7*β*,18,20,27-pentahydroxy-dammar-24-ene 20,27-bis-*O*-*β*-D-glucopyranoside, respectively.

Keywords——*Actinostemma lobatum*; Cucurbitaceae; triterpene glycoside; dammarane; (20*S*)-pentahydroxy-dammar-24-ene; (20*S*)-3*β*,6*α*,7*β*,20,27-pentahydroxy-dammar-24-ene; (20*S*)-3*β*,7*β*,18,20,27-pentahydroxy-dammar-24-ene

In the preceding paper[1] of this series, we reported the structures of four dammarane-type triterpene glycosides, actinostemmosides A (I), B (II), C (III) and D (IV), isolated from the less polar saponin fraction of the herb of *Actinostemma lobatum* MAXIM. On closer examination of the fraction by thin-layer chromatography (TLC), two new compounds, which stain violet on heating the TLC plate after spraying sulfuric acid, were detected. The less polar compound (named actinostemmoside G) has a slightly smaller *Rf* value than III, and the polar one (actinostemmoside H) is a little less mobile than actinostemoside E.[2] In addition, a dark

	R_1	R_2	R_3	R_4	R_5	R_6
I	OH	H	CH_3	*O*-*β*-Glc	CH_3	H
II	H	OH	CH_3	*O*-*β*-Glc	CH_3	H
III	H	OH	CH_2OH	*O*-*β*-Glc	CH_3	H
IV	OH	H	CH_3	CH_3	*O*-*β*-Glc$\overset{2\ 1}{—}$*α*-Rha	H
V	OH	OH	CH_3	*O*-*β*-Glc	CH_3	H
VI	H	OH	CH_2OH	*O*-*β*-Glc	CH_3	*β*-Glc

Chart 1

Table I. ^{1}H-NMR Chemical Shifts of Actinostemmosides (in Pyridine-d_5 + D_2O)

	V	VI	
H-1	0.97, 1.62	0.92, 1.65	
H-3	3.52 dd (7, 10)	3.47 dd (6, 10)	
H-5	1.28 d (11)	0.97 dd (3, 10)	
H-6	4.18 dd (9, 11)		
H-7	3.88 d (9)	4.12 dd (1, 10)	
H-9	1.45 s	1.26 s	
H-13		2.30 ddd (2, 11, 11)	
H-17	2.15 ddd (4, 10, 10)		
H-18	1.28 s	4.52 d (12), 4.70 d (12)	
H-19	0.99 s[a]	1.02 s[b]	
H-21	1.49 s	1.57 s	
H-23	2.50 m, 2.58 m	2.50 m	
H-24	5.48 t (7)	5.53 t (7)	
H-26	2.01 s	1.94 s	
H-27	4.49 d (12), 4.56 d (12)	4.62 d (12), 4.68 d (12)	
H-28	1.90 s	1.20 s	
H-29	1.39 s[a]	1.20 s[b]	
H-30	1.08 s	1.04 s[b]	
	20-*O*-Glc	20-*O*-Glc	27-*O*-Glc
G-1	5.05 d (8)	5.06 d (8)	4.87 d (8)
G-2	3.97 dd (8, 9)	3.98 dd (8, 9)	4.02 dd (8, 9)
G-3	4.23 t (9)	4.24 t[c] (9)	4.25 t[c] (9)
G-4	4.16 t (9)	4.15 t[d] (9)	4.17 t[d] (9)
G-5	3.88 ddd (9, 5, 3)	3.90 ddd (9, 6, 3)	3.95 ddd (9, 6, 3)
G-6	4.28 dd (11, 5)	4.28 dd (12, 6)	4.32 dd (12, 6)
	4.45 dd (11, 3)	4.45 dd (12, 3)	4.53 dd (12, 3)

a—d) Assignments with the same superscript may be interchanged. The numbers in parentheses are coupling constants in Hz.

Table II. ^{13}C-NMR Chemical Shifts of Aglycone Moieties[a]

C	I	V	III	VI	C	I	V	III	VI
1	39.6	39.4	39.8	39.8	16	28.2	28.1	28.2	28.2
2	27.9	27.9	28.5	28.5	17	48.4	47.6	47.4	47.6
3	78.5	78.3	78.0	78.0	18	17.7[c]	12.1	62.0	62.1
4	40.4	40.3	39.5	39.5	19	17.4[c]	17.8[e]	16.6[f]	16.6[g]
5	61.9	58.3	54.6	54.6	20	82.2	82.3	82.4	82.4
6	67.7	71.8	30.3	30.3	21	21.4	21.4	21.8	22.1
7	48.1	80.3	77.8	77.7	22	40.7	40.5	40.1	40.8
8	41.8	46.4	49.2	49.2	23	22.7	22.7	22.9	23.1
9	50.8	50.3	52.2	52.2	24	127.7	127.7	127.8	130.9
10	39.5	39.3	37.8	37.7	25	136.0	135.9	136.0	131.9
11	22.0	21.9	22.9	22.9	26	21.8	21.7	21.8	21.9
12	25.5	25.9	25.8	25.8	27	61.0	61.0	61.0	67.7
13	42.2	43.0	44.4	44.3	28	32.0	31.6	28.7	28.7
14	50.7	50.5	50.4	50.4	29	16.5[d]	16.6[e]	16.9[f]	16.8[g]
15	31.5	35.0	36.5	36.5	30	16.8[d]	16.6[b]	17.1[f]	17.1[g]

a) Spectra were measured in pyridine-d_5. Chemical shifts of the sugar carbons are given in the experimental section. Assignment was essentially based on the report by Asakawa *et al.*[5] *b*) The signal was assigned by measurement of the NOE between C_7-H and C_{14}-CH_3 and also the ^{13}C–^{1}H COSY spectrum. *c—g*) Assignments with the same superscript may be interchanged.

blue spot (lobatoside A) was observed just above the spot of actinostemmoside E. The three new compounds were isolated as described in the experimental section, and this paper deals with the structures of actinostemmosides G (V) and H (VI).

Actinostemmoside G (V) was obtained as a white powder. The fast atom bombardment mass (FAB-MS) spectrum showed the $[M+Na]^+$ ion at *m/z* 677.424, from which the molecular formula, $C_{36}H_{62}O_{10}$ was deduced. The proton-1 and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectral data (Tables I and II) suggested that V is a monohexoside of a tetracyclic triterpene.

Comparison of the ^{13}C-NMR spectrum with that of I showed that V has the same side chain and sugar moiety as I, suggesting that V is a 20-*O*-β-D-glucopyranoside of a dammarane-type triterpene. The ^{1}H- and ^{13}C-NMR signals of the sugar moiety were distinguished and unambiguously assigned as those of β-D-glucopyranose in the C1 conformation by measurement of ^{1}H–^{1}H and ^{13}C–^{1}H correlation spectroscopy (COSY) spectra and from the chemical shifts and splitting patterns of protons and the coupling constant (156 Hz) of the anomeric carbon and proton.[3] From these data, seven oxygenated carbons were assigned, and thus, the remaining three hydroxymethine carbons should be in the dammarane nucleus. The ^{1}H-NMR signals of the three protons appeared at δ 3.52 (dd, J=7, 10 Hz), δ 3.88 (d, J=9 Hz) and δ 4.18 (dd, J=9, 11 Hz). The first one was assigned as the C_3-H by comparison with the ^{1}H-NMR spectrum of I. The second proton is on a carbon next to a quaternary carbon and couples with the third hydroxymethine proton, which further couples with a methine proton (δ 1.28, d, J=11 Hz) adjacent to two quaternary carbons. The two hydroxyl groups were reasonably allocated at C_6 and C_7, and the upfield shifts of the ^{13}C-NMR signals assigned to C_{18} (Δ 5.6 ppm) and C_5 (Δ 3.6 ppm), and down field shifts of the signals of C_{15} (Δ 3.5 ppm), C_8 (Δ 4.6 ppm), C_7 (Δ 32.2 ppm) and C_6 (Δ 4.1 ppm) compared to those of I are well rationalized on this basis. The coupling constants of protons on C_5, C_6 and C_7 indicated that the orientations of the hydroxyl groups at C_6 and C_7 are α and β, respectively. The presence of the nuclear Overhauser effect (NOE) between C_7-H and both C_5-H and one methyl group (C_{14}-CH_3) supports this orientation.

All these spectral data led to the conclusion that V is (20*S*)-3β,6α,7β,20,27-pentahydroxy-dammar-24-ene 20-*O*-β-D-glucopyranoside.

Actinostemmoside H (VI) was obtained as colorless needles and was analyzed as $C_{42}H_{72}O_{15}\cdot 1.5H_2O$. Acid hydrolysis and gas liquid chromatography (GLC) examination[4] of the sugar fraction revealed the component sugar to be D-glucose. The ^{1}H- and ^{13}C-NMR spectral data suggested that VI is a bis-hexoside of a dammarane-type triterpene having one tri-substituted double bond, one tertiary hydroxyl group, two secondary hydroxyl groups and two primary hydroxyl groups. By detailed examination of the ^{13}C- and ^{1}H-NMR spectra and from the coupling constants of anomeric carbons and protons (160 and 156 Hz), each hexosyl group was assigned as β-D-glucopyranose in the C1 conformation.

The ^{13}C-NMR spectrum of VI was compared with that of III. The spectra of the aglycone moieties were quite similar, except that the signals of C_{27} and C_{24} of III were shifted downfield by 6.6 and 3.1 ppm, respectively, and the signal of C_{25} was shifted upfield by 4.1 ppm in the case of VI. These data clearly indicated that VI is a 27-*O*-β-D-glucopyranoside of III, *viz.* (20*S*)-3β,7β,18,20,27-pentahydroxy-dammar-24-ene 20,27-bis-*O*-β-D-glucopyranoside.

Lobatoside A, $C_{41}H_{66}O_{14}\cdot 2.5H_2O$, was obtained as colorless needles. The molecular formula implies that lobatoside A is a triterpene glycoside having 1 mol each of a hexose and a pentose. The coloration of the spot on TLC suggests it to be a less polar saponin having a common aglycone with that of the polar saponins which were named lobatosides.[1] The structure of lobatoside A will be discussed in a subsequent report in connection with other lobatosides.

Experimental[6]

Isolation of V, VI and Lobatoside A——The mother liquor (10 mg) after crystallization of III was chromatographed on silica gel [solvents: $CHCl_3$–MeOH–H_2O (9 : 3 : 0.5), AcOEt-PrOH–H_2O (15 : 3 : 0.5)] and on LiChroprep RP-18 (65% MeOH) and then subjected to high performance liquid chromatography (HPLC) successively on a reversed-phase column (Shim-Pack CLC-ODS) and a normal-phase column (Shim-Pack CLC-SIL), resulting in the isolation of V (5 mg) as a white powder. FAB-MS *m/z*: 677.424 ([M+Na]$^+$). $C_{36}H_{62}NaO_{10}$ requires *m/z* 677.424. ^{1}H-NMR: shown in Table I. ^{13}C-NMR: sugar moiety; 98.6 (1), 75.6 (2), 79.0 (3), 71.9 (4), 77.9 (5), 63.0 (6).

The fraction (300 mg) less polar than actinostemmoside E obtained from fr.4b-1[1] was chromatographed repeatedly on silica gel [solvent: AcOEt–MeOH–H_2O (35 : 4 : 3), $CHCl_3$–MeOH–H_2O (9 : 3 : 0.5) and AcOEt–PrOH–H_2O (5 : 1 : 0.5)] to give lobatoside A (250 mg): colorless needles from aqueous MeOH, mp 249—252 °C. $[\alpha]_D^{24}$49.4° (c=0.53, MeOH). *Anal.* Calcd for $C_{41}H_{66}O_{14} \cdot 2.5H_2O$: C, 59.63; H, 8.66. Found: C, 59.47; H, 8.64. FAB-MS *m/z*: 805 ([M+Na]$^+$), 781 ([M−H]$^-$).

The fraction (80 mg) more polar than actinostemmoside E was chromatographed in the same manner to give VI (70 mg): colorless needles from aqueous MeOH, mp 135—140 °C. $[\alpha]_D^{24}$+1.6° (c=0.54, MeOH). *Anal.* Calcd for $C_{42}H_{72}O_{15} \cdot 1.5H_2O$: C, 59.83; H, 9.22. Found: C, 59.77; H, 8.96. FAB-MS *m/z*: 839 ([M+Na]$^+$), 815 ([M−H]$^-$). ^{1}H-NMR: shown in Table I. ^{13}C-NMR: 20-*O*-glucosyl moiety; 98.6 (1), 75.6 (2), 78.8[7a] (3), 71.8[7b] (4), 77.8 (5), 62.9 (6), 27-*O*-glucosyl moiety; 103.3 (1), 75.1 (2), 78.5[7a] (3), 71.7[7b] (4), 78.4 (5), 62.8 (6).

Identification of the Component Monosaccharide of VI——Compound VI (3 mg) was dissolved in 2 N H_2SO_4 (1 ml) and heated in a boiling water bath for 4 h. The reaction solution was neutralized with Amberlite IR-410 and lyophilized. The residue was treated as described by Hara *et al.*[4] and the trimethylsilyl ether of the thiazolidine derivative of the component sugar was checked by GLC.[8] The hydrolysate of VI gave one peak with a t_R value (16.71 min) identical with that of D-glucose (t_R value of L-glucose derivative, 17.64 min).

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6) The instruments and materials used in this work are as follows: Yazawa micromelting point apparatus, JASCO DIP-4 digital polarimeter, JEOL JNM GX-400 spectrometer (100 MHz for ^{13}C-NMR spectra and 400 MHz for ^{1}H-NMR spectra), JEOL JMS DX-300 mass spectrometer, Shimadzu liquid chromatograph LC-6A, Shimadzu gas chromatograph GC-8A, Kieselgel 60 (63—210 μm, E. Merck), Daisogel IR-60 (40—63 μm, Osaka Soda Co., Ltd.), LiChroprep RP-18 (40—63 μm, packed column, 31 × 2.5 cm i.d., E. Merck) and LiChroprep Si60 (40—63 μm, packed column, 44 × 3.7 cm i.d., E. Merck), precoated Kieselgel 60 F_{254} plates, precoated HPTLC RP-18 F_{254} plates (E. Merck), Shim-Pack CLC-ODS (5 μm) and Shim-Pack CLC-SIL (5 μm) (Shimadzu). ^{1}H-NMR spectra were measured in pyridine-d_5 containing D_2O and ^{13}C-NMR spectra were obtained in pyridine-d_5 solutions. Chemical shifts are expressed on the δ scale with tetramethylsilane as an internal standard. The FAB-MS spectra were obtained in a glycerol matrix containing NaI.
7) Definite assignment could not be done. Assignments with the same superscript may be interchanged.
8) The GLC conditions were as follows: column, G-SCOT OV-17 on Silanox (0.3 mm i.d. × 50 m); column bath temperature, 210 °C; injection temperature, 270 °C; carrier gas, He (0.75 ml/min); split ratio, 1/75; make-up gas, He (50 ml/min).

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Reaction of Cyclic Thioxocarbonates with Tributyltin Hydride[1)]

KIMIHIRO KANEMITSU,[a] YOSHISUKE TSUDA,*[,a] MOHAMMED EKRAMUL HAQUE,[b] KOJI TSUBONO,[b] and TOHRU KIKUCHI*[,b]

Faculty of Pharmaceutical Sciences, Kanazawa University,[a] 13-1 Takara-machi, Kanazawa 920, Japan and Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University,[b] 2630 Sugitani, Toyama 930-01, Japan

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Treatment of methyl 2-*O*-acetyl-3,4-*O*-thiocarbonyl-β-L-arabinopyranoside with tributyltin hydride produced seven compounds, depending on the reaction conditions: the deoxy derivatives (**2** and **3**), the methylidene derivative (**4**), the carbonate (**5**), the *O*,*S*-rearrangement products (**7** and **8**), and the 4-deoxy acetyl migration product (**9**). Their structures were determined by spectroscopic and chemical means, and their formation mechanisms are discussed.

Keywords——cyclic thioxocarbonate; tributyltin hydride; radical deoxygenation; *O*,*S*-rearrangement; oxygen trap; acyl migration

Previously, in deoxygenation studies of non-protected glycopyranosides,[1b,2)] we have noticed that cyclic thioxocarbonates gave, in some instances, several by-products together with the expected deoxy compounds when treated with Bu_3SnH in the presence of α,α'-azobisisobutyronitrile(AIBN).[1b)] Although several reports on the Bu_3SnH deoxygenation of cyclic thioxocarbonates have appeared,[3)] none of them referred to the side reactions. In this paper, we describe the details of the structure determination of the by-products and discuss their formations, based on methyl 2-*O*-acetyl-3,4-*O*-thiocarbonyl-β-L-arabinopyranoside **1** as a model compound.

The ordinary procedure of deoxygenation of **1** is as follows: **1** is treated with Bu_3SnH (1—1.5 mol eq) and a catalytic amount of AIBN (*ca.* 0.1 mol eq) in toluene at 75 °C for 30 min under an argon atmosphere. A typical gas liquid chromatography (GLC) pattern of the reaction mixture thus produced showed six peaks (Fig. 1). Four spots were seen on thin layer chromatography (TLC) (not shown). These spots were separated by preparative high performance liquid chromatography (HPLC).

The major product (peaks A and B), obtained in 56% yield, corresponded to the least mobile spot, and was a mixture of the previously reported 4-deoxy and 3-deoxy compounds (**2** and **3**).[1b,2)] The ratio was determined from the peak heights in GLC and the structures were determined on the basis of COSY and ^1H–^{13}C COSY spectra of the mixture. The signals were fully assigned, and the structure of each compound was established.

The compound corresponding to peak C (16%) is the most mobile on TLC. It had the formula $C_9H_{14}O_6$. In the proton-nuclear magnetic resonance (^1H-NMR) spectrum, it showed signals at δ 5.00 and 5.21 corresponding to two protons with the coupling constant of $J=$ 1 Hz, indicating the presence of an O–CH_2–O group. The carbon signal at δ 94.8 confirmed this. Since the other signals were assigned as in the arabinoside, the compound was concluded to be the methylidene derivative **4**.

The compound corresponding to peak D (10%) gave a spot close to **1** on TLC and was isolated as colorless needles, mp 119 °C. It exhibited an infrared (IR) absorption at

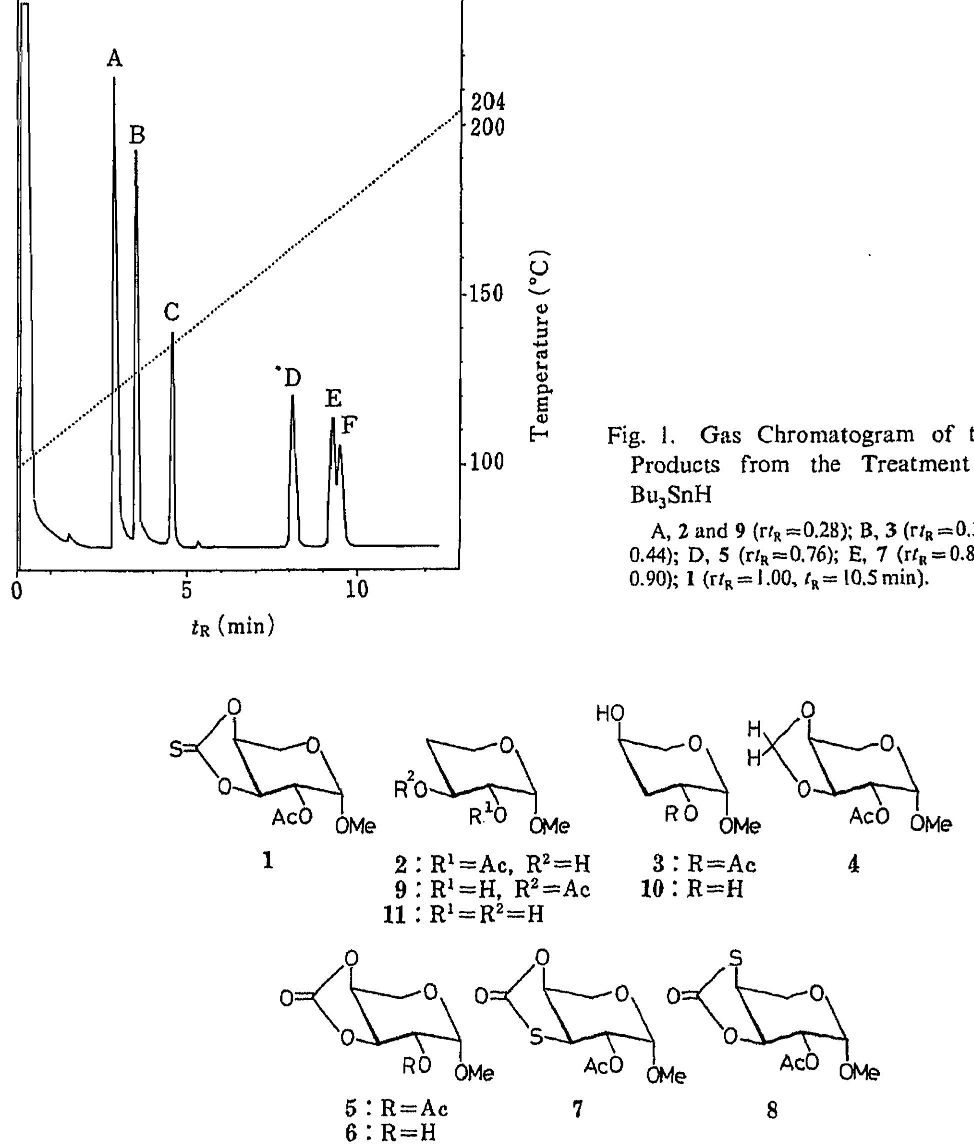

Fig. 1. Gas Chromatogram of the Reaction Products from the Treatment of **1** with Bu_3SnH

A, **2** and **9** (rt_R=0.28); B, **3** (rt_R=0.33); C, **4** (rt_R=0.44); D, **5** (rt_R=0.76); E, **7** (rt_R=0.89); F, **8** (rt_R=0.90); **1** (rt_R=1.00, t_R=10.5 min).

Chart 1

$1813\,cm^{-1}$, in addition to that due to the OAc group ($1740\,cm^{-1}$), suggesting the presence of a five membered carbonate group. The corresponding carbonyl signal in the ^{13}C-NMR spectrum was difficult to observe by usual measurement. However, use of the homo-gated decompling technique clearly indicated the peak at δ 154.4. Thus, the compound was assigned as the carbonate **5**. The structure was confirmed by acetylation of the carbonate **6** prepared by an alternative synthesis.[4)]

The compounds corresponding to peaks E and F showed the same *Rf* value as **1** on TLC. These were obtained in 15% yield as a mixture, from which compound E crystallized out as prisms, mp 149—151 °C, on keeping the mixture in hexane–EtOAc. Compound F was a syrup.

Compound E had the same formula, $C_9H_{12}O_6S$, as the thioxocarbonate **1** and exhibited an IR absorption at $1760\,cm^{-1}$, in addition to that of the acetyl group ($1740\,cm^{-1}$), which also gave a carbon-13 nuclear magnetic resonance (^{13}C-NMR) signal at δ 171.5. It also showed signals at δ 4.35 (1H, dd, J=4.9 and 10.7 Hz) in the ^1H-NMR spectrum and at 47.0 (d) in the ^{13}C-NMR, both of which are indicative of the presence of an –S–CH– group. The

position of the S–C linkage at C-3 was determined by means of COSY and $^{1}H-^{13}C$ COSY experiments. Thus, this compound is the 3-thiol-3,4-carbonate **7**.

The other compound F also had the formula $C_9H_{12}O_6S$ as shown by gas chromatography-mass spectrometry (GC-MS). It showed –S–CH– signals at δ 4.71 (1H, ddd, J=6.5, 3.6 and 2.4 Hz) in the ^{1}H-NMR and at δ 48.2 (d) in the ^{13}C-NMR spectra, suggesting that it is the 4-thiol-3,4-carbonate **8**. The assignment was supported by a COSY experiment.

In agreement with the above assignments, desulfurization of compounds E and F with Raney Ni followed by alkaline treatment produced the 3-deoxy and 4-deoxy compounds (**10** and **11**), which were identical with the deacetylation products obtained from **3** and **2**, respectively. The compounds **7** and **8**, therefore, are the *O,S*-rearrangement products.

On elevating the reaction temperature to 100 °C (Table II, entry 4), the seventh compound was produced. Although this compound could be separated on TLC, it was inseparable from the 4-deoxy compound **2** on GLC. The ^{1}H- and ^{13}C-NMR spectra indicated that it is the 4-deoxy-3-*O*-acetyl derivative **9**, since C^3-H and C^2-H appeared at δ 5.56 (1H, dt, J=5.0 and 10.5 Hz) and δ 4.00 (1H, dd, J=4.0 and 10.0 Hz), respectively, in a COSY experiment. It was hydrolyzed to the same 4-deoxy compound **11** obtained from **2**. Hence, it is the acetyl migration product **9** formed from **2**. Such an easy acyl migration between diequatorially disposed *O*-2 and *O*-3 hydroxyl groups under thermal conditions has already been reported in D-glucopyranosides.[5]

Next we will briefly discuss the reaction mechanism. Plausible routes to the above products are illustrated in Chart 2. According to the known mechanism of tin hydride deoxygenation,[6] formation of the deoxy compound **e** from a cyclic thioxocarbonate **a** can be explained by breakdown of the initial radical **b** to the rearranged radical **c** followed by hydride radical trapping. The real species in the reaction mixture may be **d**, which decomposes into **e** during the isolation procedure, since direct injection of the reaction mixture into a GLC column did not reveal peaks of **2** and **3**, which appeared after passing the mixture through a silica gel column.

When a large excess of tin hydride is present, the radical **b** is trapped by a hydride radical before rearrangement, thus leading to the methylidene derivative **f**. In fact, **4** became the major product (75%), when the reaction of **1** was carried out with 5 mol eq of Bu_3SnH (Table II, entry 5).

The radical **b** can also be trapped by oxygen, leading to the carbonate **g**. This process

TABLE I. ^{13}C-NMR Data for **1**, **4**, **5**, **6**, **7**, and **8** in Pyridine-d_5[a]

Compd.	C-1	C-2	C-3	C-4	C-5	OMe	$-CH_3$	C=O	Others
1	96.3	70.2	78.7	80.5	57.0	55.7	20.4	169.7	191.5 (O,O>C=S)
4	97.6	71.7	72.7	75.1	58.6	55.4	20.8	170.5	94.8 (O,O>CH_2)
5	96.6	70.8	74.7	75.9	57.7	55.8	20.5	169.9	154.4 (O,O>C=O)
6	99.6	69.0	78.2	76.3	58.3	55.7			155.2 (O,O>C=O)
7	96.3	73.5	47.0	80.6	57.5	55.5	20.5	170.1	171.5 (O,S>C=O)
8	97.5	69.3	78.0	48.2	57.3	55.6	20.5	170.1	171.6 (S,O>C=O)

a) Assignments were confirmed by $^{1}H-^{13}C$ COSY experiments.

Chart 2

seems to be faster than the others for the following reasons; i) no oxygenated product (such as Me β-L-Ara or Me α-D-Xyl) derived from the radical **c** was observed, ii) the ratio of the carbonate **5** increased when stored Bu_3SnH was used (Table II, entry 2), and iii) it was produced exclusively when the reaction of **1** was carried out with bubbling air. The use of freshly distilled Bu_3SnH can minimize the formation of **g**, though we could not completely eliminate it.

The rearranged radical **c** can cyclize to **h**, the *O,S*-rearrangement product, with regeneration of a tin radical. Interestingly, this process suggests that the tin radical acts as a catalyst for the formation of **h**.[7] The rearrangement product **h** can again be reduced by tin hydride to the deoxy species **d** thus giving rise to **e**. Actually, further treatment of **7** with an excess of Bu_3SnH and a catalytic amount of AIBN afforded the deoxy compound **3**, suggesting that the process, **c** to **h**, is reversible.

Experimental

Melting points were determined on a Yanagimoto micro hot stage apparatus and are uncorrected. IR spectra were taken in chloroform on a Jasco IR A202 spectrometer and are given in cm^{-1}. The ^1H- and ^{13}C-NMR spectra were taken at 400 and 100 MHz, respectively, on a JNM GX-400 FT NMR spectrometer in pyridine-d_5 with tetramethylsilane as an internal reference. GLC was carried out with a Shimadzu GC4CM-PF gas chromatograph coupled with an FID detector, using a glass column (1 m × 3 mm i.d.) packed with 1.5% OV-1 on Shimalite W (80—100 mesh), with N_2 (50 ml/min) as a carrier gas. A linear temperature rise at 8 °C/min was adopted. GC-MS was done on a JEOL JMS-D 300 spectrometer. Column chromatography and preparative HPLC were performed on silica gel (Fujigel BW-820 MH) and Silica gel Si-60 (Merck), respectively. For TLC, precoated plates (Machery-Nagel SIL G-25, 0.25 mm thick) were used with benzene–EtOAc (1 : 2) as the developing solvent, and spots were observed by spraying 1% $Ce(SO_4)_2$ in 10% H_2SO_4 followed by heating.

Reaction of Methyl 2-*O*-Acetyl-3,4-*O*-thiocarbonyl-β-L-arabinopyranoside 1 with Bu_3SnH——i) Ordinary Procedure for Deoxygenation (Table II, Entry 1): A solution of **1** (300 mg) and AIBN (20 mg, 0.1 mol eq) in toluene (50 ml) was warmed at 75 °C until the compounds completely dissolved. To this solution, freshly distilled Bu_3SnH (352 mg, 1 mol eq) was added by injection and the mixture was stirred for 30 min at 75 °C under an argon atmosphere. The reaction mixture was directly subjected to column chromatography on silica gel; elution was carried out with benzene, then EtOAc. Concentration of the EtOAc eluate gave a mixture of products (GLC shown in Fig. 1),

TABLE II. Product Distributions in the Reaction of **1** with Bu_3SnH

Entry	Molar ratio		Reaction conditions[a]		Product (yield, %)[b]			
	Bu_3SnH	AIBN	(°C)	(h)	Deoxy compds. (**2** and/or **9**:**3**)	*O,S*-Rearr. compds. (**7**:**8**)	Others **4**	**5**
1	1	0.1	75	0.5	56 (7:5)	15 (3:2)	16	10
2	1[c]	0.1	75	0.5	25 (6:4)	13 (5:3)	—	62
3	1.5	0.2	75	1	52 (7:5)	13 (7:5)	25	10
4	1.5	0.2	100	1	44 (4:1)	15 (5:4)	28	13
5	5	0.2	100	1	11 (1:1)	Trace	75	10

a) Reaction of 50—300 mg of 1. *b*) Yields from GLC for entries 2 and 3; the others are isolated yields calibrated by GLC. The ratios (in parentheses) were calculated from GLC peak areas. *c*) Stored Bu_3SnH was used.

which was separated by HPLC [solvent: benzene–EtOAc (1:1)]. The methylidene derivative **4** (42 mg), syrup, was first eluted and then a mixture of the *O,S*-rearrangement products (45 mg), **7** and **8**, from which the 3-thiol-3,4-carbonate **7** (27 mg), mp 149—151 °C, crystallized out when the mixture was kept in an appropriate amount of hexane–EtOAc. The next eluate contained the carbonate **5** (28 mg), mp 117—119 °C, which readily crystallized from hexane–EtOAc, and finally the deoxy compounds (130 mg), **2** and **3**, were eluted as a mixture.

ii) At Elevated Temperature (Table II, Entry 4): Compound **1** (100 mg) was allowed to react under the conditions described in entry 4 and worked up as above. The reaction mixture gave the following compounds; **4** (25 mg), a mixture of **7** and **8** (15 mg), **5** (12 mg), and a mixture of deoxy compounds (35 mg). The last fraction was further separated by repeated HPLC to give **9** (22 mg) and **3** (10 mg) contaminated with **2**.

iii) With Excess Hydride (Table II, Entry 5): Compound **1** (80 mg) was allowed to react as described in entry 5 to give the following compounds; **4** (52 mg), **5** (7.5 mg), and a mixture of **9** and **3** (6.8 mg).

The results of reactions under various conditions including the above are summarized in Table II.

Physical and spectral data for compounds **2**—**5** and **9** were given in the previous paper,[1b] and those of **7** and **8** are summarized below.

Methyl 2-*O*-Acetyl-3,4-*S*,*O*-carbonyl-3-deoxy-3-thiol-β-L-arabinopyranoside 7——Colorless needles from hexane–EtOAc, mp 149—151 °C. ^{1}H-NMR: 5.27 (1H, dd, J=3.0, 10.7 Hz, H-2), 5.13 (1H, d, J=3.0 Hz, H-1), 4.96 (1H, dd, J=2.4, 4.9 Hz, H-4), 4.35 (1H, dd, J=4.9, 10.7 Hz, H-3), 4.15 (1H, d, J=13.8 Hz, H-5_{eq}), 3.93 (1H, dd, J= 2.4, 13.8 Hz, H-5_{ax}), 3.32 (3H, s, CH_3), 2.02 (3H, s, $COCH_3$), IR: 1760, 1740. GC-MS *m/z*: 248 (M^+), 217 ($M^+ - OCH_3$), 188 ($M^+ - CH_3COOH$). *Anal.* Calcd for $C_9H_{12}O_6S$: C, 43.55; H, 4.87; S, 12.89. Found: C, 43.53; H, 4.89; S, 12.86.

Methyl 2-*O*-Acetyl-3,4-*S*,*O*-carbonyl-4-deoxy-4-thiol-β-L-arabinopyranoside 8——Syrup. ^{1}H-NMR: 5.61 (1H, dd, J=3.1, 8.5 Hz, H-2), 5.22 (1H, dd, J=8.5, 6.5 Hz, H-3), 5.12 (1H, d, J=3.1 Hz, H-1), 4.71 (1H, ddd, J=6.5, 3.6, 2.4 Hz, H-4), 4.15 (1H, dd, J=3.6, 13.4 Hz, H-5_{eq}), 3.98 (1H, dd, J=2.4, 13.4 Hz, H-5_{ax}), 3.34 (3H, s, CH_3), 1.98 (3H, s, $COCH_3$). IR: 1762, 1740. GC-MS *m/z*: 248 (M^+), 217 ($M^+ - OCH_3$), 188 ($M^+ - CH_3COOH$).

Bu_3SnH Treatment of 1 with Bubbling Air——Compound 1 (50 mg) and AIBN (0.1 mol eq) was treated with Bu_3SnH (1 mol eq) in toluene (10 ml) under bubbling air at 75 °C for 30 min and worked up as described above. The gas chromatogram of the EtOAc fraction (42 mg) showed a single peak of the carbonate **5**.

Methyl 2-*O*-Acetyl-3,4-*O*-carbonyl-β-L-arabinopyranoside 5 from 6——Acetylation of Me β-L-Ara 3,4-carbonate **6**[4] (10 mg) with Ac_2O (0.7 ml)–pyridine (1 ml) at room temperature for 12 h gave, after usual work-up, the 2-*O*-acetate **5**. This product was identical with the 3,4-*O*-carbonate obtained above.

Raney Ni Desulfurization of 7 and 8——i) Compound **7** (10 mg) was stirred with an excess of Raney Ni in MeOH (2 ml) for 12 h at room temperature. Methanolysis of the product with CH_3ONa in MeOH, followed by neutralization with Amberlite IR-120-H^+, gave the 3-deoxy derivative **10** (5.5 mg), which was identical with the compound obtained by methanolysis of **3** in TLC and GLC comparisons.

ii) Similar desulfurization followed by methanolysis of **8** (containing a small amount of **7**) gave the 4-deoxy derivative **11**, which was identical with the compound obtained below.

Methanolysis of 2 and 9——Cmpounds **2** and **9** (each 10 mg) were treated with CH_3ONa in MeOH for 1 h. Neutralization of the mixture with Amberlite IR 120-H^+ and evaporation of the solvent gave the same 4-deoxy derivative **11** (7.2 and 7.0 mg, respectively) as a gum. Its identity was confirmed by TLC and GLC.

Treatment of 7 and 8 with Bu_3SnH——i) A mixture of **7** (10 mg), a catalytic amount of AIBN and Bu_3SnH (5 mol eq) in toluene (2 ml) was stirred for 30 min at 75 °C under an argon atmosphere. The reaction mixture was directly passed through a short silica gel column with benzene, then EtOAc. GLC of the EtOAc eluate (7.3 mg) exhibited a single peak (B) corresponding to the 3-deoxy derivative **3**.

ii) A mixture of **8** and **7** (8 : 2) was similarly treated with Bu_3SnH in toluene to give a gum; GLC showed two peaks (A and B) corresponding to **2** and **3** (7 : 2).

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Studies on Peptides. CLV.[1,2] Evaluation of Trimethylsilyl Bromide as a Hard-Acid Deprotecting Reagent in Peptide Synthesis

NOBUTAKA FUJII, AKIRA OTAKA, NAOKI SUGIYAMA, MIKA HATANO, and HARUAKI YAJIMA*

Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan

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Trimethylsilyl bromide (TMSBr) in trifluoroacetic acid (TFA) was found to have the ability to cleave benzyl-type protecting groups, *i.e.*, benzyloxycarbonyl (Z), benzyl (O-Bzl) and *p*-methoxybenzyl (S-MBzl). The reaction was best accelerated by addition of thioanisole, compared with other soft nucleophiles so far examined. The rate of the cleavage reaction with TMSBr/TFA was judged to be somewhat slower than that with trimethylsilyl trifluoromethanesulfonate/TFA. However, TMSBr/TFA reduced Met(O) efficiently and gave almost no side reaction of Asp (succinimide formation). This deprotecting procedure was applied to the synthesis of human gastrin-releasing peptide.

Keywords——trimethylsilyl bromide; hard acid deprotecting reagent; soft nucleophile; thioanisole; trimethylsilyl trifluoromethanesulfonate; trifluoromethanesulfonic acid; Met sulfoxide; Asp-side reaction; human gastrin-releasing peptide

Removal of Bzl-type protecting groups by HBr/AcOH was first demonstrated by Ben-Ishai and Berger[3] in 1952. This deprotecting procedure was applied to the synthesis of α-melanocyte-stimulating hormone by Guttmann and Boissonnas[4] in 1958 and later, to the solid phase peptide synthesis by Merrifield[5] in 1963. However, this procedure did not gain wide acceptance in practical peptide synthesis, since HF was found to be useful for the same purpose by Sakakibara *et al.*[6] in 1967. From a practical standpoint, preparation and handling of HBr/AcOH reagent are rather laborious. If the HF deprotecting procedure had not been available, presumably the solid phase synthesis would not have gained its present degree of applicability. However, in spite of the attractive features of the HF procedure, the HBr/TFA deprotecting procedure still appears to be superior to HF for suppressing acid-catalyzed succinimide formation of the Asp residue linked to particular amino acids, as reported by Bodanszky *et al.*[7]

We wish to report that Bzl-type protecting groups can be cleaved, as by HBr/TFA, when the trimethylsilyl group is supplied as a source of hard acid, in lieu of the proton of HBr in TFA, and thioanisole is used as a soft nucleophile.[8] The efficiency of 1 M TMSBr–thioanisole/TFA as a peptide deprotecting reagent was examined and the results were compared with those of 1 M TMSOTf–thioanisole/TFA.[9]

Amino acid derivatives were treated with 1 M TMSBr/TFA in the presence of various cation scavengers in an ice-bath for 60 min and amino acids regenerated were determined quantitatively by using an amino acid analyzer. As shown in Table I, in this system, thioanisole was found to be most effective to accelerate the cleavage reaction, compared with other scavengers examined. The Bzl group was cleaved from Ser, Thr, Glu and Asp and the Z group from Lys in nearly quantitative yields by 1 M TMSBr–thioanisole/TFA. In addition, the Cl_2–Bzl group[10] from Tyr and the Mts group[11] from Trp and Arg were cleaved in nearly

TABLE I. Removal of Various Protecting Groups by 1 M Me_3SiBr/TFA in the Presence of a Soft Base

Treated amino acid deriv. (0 °C, 60 min)	Parent amino acid regenerated (%)			
	Anisole	Thioanisole	MeSMe	PhSPh
Z(OMe)–Lys(Z)–OH		87.8		
Z(OMe)–Ser(Bzl)–OH	64.1	85.7	82.3	82.3
Boc–Thr(Bzl)–OH		100.0		
Z(OMe)–Glu(OBzl)–OH		93.9		
Boc–Asp(OBzl)–OH	27.6	100.5	45.2	46.0
Boc–Asp(OChp)–OH		4.2	3.9	5.0
Boc–Tyr(Cl_2–Bzl)–OH	32.9	96.9	74.7	76.2
Boc–Trp(Mts)–OH	3.3	86.1	13.6	11.4
Z(OMe)–Arg(Mts)–OH	23.4	87.0	19.0	40.8
H–Cys(MBzl)–OH		98.7		
Z(OMe)–Cys(Ad)–OH		0		
Boc–Cys(*tert*-Bu)–OH		7.6		
Z(OMe)–Met(O)–OH		91.3		

90% yield under these conditions. However, removal of the Chp group[12] from Asp was unsuccessful. Of various S-protecting groups of cysteien, the MBzl group[13] was cleaved completely, but others were not cleaved under the conditions stated above. Thus, the rate of cleavage by 1 M TMSBr–thioanisole/TFA was judged to be much slower than that by 1 M TMSOTf–thioanisole/TFA. However, it seems worthwhile to note that Met(O)[14] was reduced back to Met in 91% yield by this TMSBr/TFA system, while the 1 M TMSOTf–thioanisole/TFA system reduced Met(O) in only 27% yield under identical conditions.

Next, Asp-succinimide formation by this deprotecting reagent was examined and the result was compared with that in the case of 1 M TMSOTf/TFA. A model peptide, Z(OMe)–Ala–Asp(OBzl)–Gly–OBzl[12] was treated with 1 M TMSBr/TFA or 1 M TMSOTf/TFA in the presence of thioanisole or diphenylsulfide in an ice-bath for 120 min and the succinimide formed was quantitated with a dual-wavelength TLC (thin-layer chromatography) scanner. The amount of the side product formed by TMSBr/TFA treatment was judged to be negligible, while TMSOTf/TFA treatment gave the side product in 4.6% yield. In this respect, the TMSBr/TFA system seems to be superior to the TMSOTf/TFA system. The deprotecting procedure based on the hard acid principle was found not to be entirely free from the ring closure reaction of the Asp residue, depending on the nature of hard acid employed, as we had observed in human pancreatic polypeptide synthesis.[1]

The above results suggested that this TMSBr/TFA deprotecting procedure can be applied to the synthesis of relatively small peptides containing Asp or Met. As a model experiment, protected human gastrin releasing polypeptide (hGRP)[15] containing two Met(O) residues was deblocked by treatment with TMSBr/TFA. In the previous synthesis, these Met(O) residues were reduced by treatment with phenylthiotrimethylsilane,[16] prior to TFMSA/TFA deprotection.[17] However, in the present experiment, protected hGRP was directly treated with 1 M TMSBr–thioanisole/TFA in an ice-bath for 3 h to ensure the complete deprotection of the three protecting groups, Z from Lys and two Mts groups from Arg and Trp, and at the same time the complete reduction of the two Met(O). The crude product obtained after gel-filtration was examined by high-performance liquid chromatography (HPLC). As shown in Fig. 1, the product possessing an identical retention time with that of an authentic sample of hGRP emerged from the column as the main component. The desired product was isolated by preparative HPLC in 52.4% yield. Thus, the desired peptide

Z(OMe)–Val–Pro–Leu–Pro–Ala–Gly–Gly–Gly–Thr–Val–Leu–Thr–Lys(Z)–Met(O)–
Tyr–Pro–Arg(Mts)–Gly–Asn–His–Trp(Mts)–Ala–Val–Gly–His–Leu–Met(O)–NH_2
↓ 1) 1 M TMSBr–thioanisole/TFA
2) gel-filtration on Sephadex G-25
crude hGRP
↓ 3) HPLC
purified hGRP

a) b)

0 10 20 0 10 20 30

Fig. 1. HPLC of the Crude Sample of Synthetic hGRP

a) Gel-filtered sample. b) Gel-filtered sample (20 μg)+synthetic hGRP[15] (25 μg).

was obtained in a much better yield than before (30%), without particular treatment for the reduction of Met(O), before or after deprotection.

Some advantageous features of the 1 M TMSBr–thioanisole/TFA deprotecting procedure have thus been demonstrated. We intend to evaluate further whether this hard acid deprotecting procedure with TMSBr can be applied to the synthesis of more complex peptides by increasing its concentration in TFA.

Experimental

Amino acid analysis and HPLC were conducted with a Hitachi 835-02 instrument and a Waters 204 compact model, respectively. Optical rotation and ultraviolet absorption (UV) were determined with Union PM-101 and Hitachi 100-20 instruments, respectively. *Rf* values in TLC on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 *n*-BuOH–pyridine–AcOH–H_2O (3 : 1 : 1 : 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_3 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1), and the ninhydrin color intensity was measured with a Shimadzu dual-wavelength TLC scanner, model CS-900. Leucine-aminopeptidase was purchased from Sigma Chem. Co. (Lot. No. 62F-8000).

Treatment of Amino Acid Derivative with 1 M TMSBr/TFA in the Presence of Various Scavengers——A mixture of an amino acid derivative (0.03 mmol each) and Gly (internal standard) was treated with 1 M TMSBr/TFA (0.6 ml, reagent 20 eq each) in an ice-bath for 60 min. As a scavenger, four compounds were tested: anisole, thioanisole, MeSMe (to a concentration of 1 M for each), and PhSPh (not freely soluble, but an amount equivalent to 1 M was added). A part of the solution was subjected to quantitative amino acid analysis and the results are listed in Table I.

Treatment of Z(OMe)–Ala–Asp(OBzl)–Gly–OBzl with 1 M TMSBr–Thioanisole/TFA——A sample (10 mg) was treated with 1 M TMSBr–thioanisole/TFA (0.4 ml) in an ice-bath for 120 min. The product was precipitated with *n*-hexane and examined by TLC with the scanner using authentic samples of H–Ala–Asp–Gly–OH (Rf_1 0.20) and H–Ala–Asc–Gly–OH (Rf_1 0.33) as references.

Deprotection of Protected hGRP by Treatment with 1 M TMSBr–Thioanisole/TFA——Protected hGRP (50 mg, 15.7 μmol) was treated with 1 M TMSBr–thioanisole/TFA (5 ml) in the presence of *m*-cresol (16.7 μl, 10 eq per Tyr) and EDT (13.4 μl, 10 eq per Trp) in an ice-bath for 3 h, then dry ether was added. The resulting powder was dissolved in H_2O (2 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH and 1 M NH_4F (200 μl) was added. After 30 min, the pH was readjusted to 5.0 with 1 N AcOH and the solution was applied to a column of Sephadex G-25 (2.3 × 130 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak (tube Nos. 36—50, 6 ml each, determined by UV absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization to give a crude deprotected peptide; yield 41 mg (94%). Its HPLC elution pattern on an ODS column (AM-302, Yamamura Chemical Co.) with 25% MeCN in 0.1% TFA is shown in Fig. 1a, in

comparison with that of a mixture of the crude sample and an authentic sample of synthetic hGRP (Fig. 1b).

The product (9.1 mg) was purified by HPLC using a Nucleosil 5C18 column (10 x 250 mm) with isocratic elution with MeCN (27%) in 0.1% TFA. The desired eluate (retention time 24 min) was collected, then the solvent was removed by evaporation and the residue was lyophilized to give a fluffy white powder. The rest of the sample was similarly purified; yield 23.6 mg (52.4% from the protected hGRP), Rf_2 0.45, Rf_3 0.48, $[\alpha]_D^{21} -99.7°$ (c=0.1, 1 N AcOH), (lit.[15] −99.9 °C in 1 N AcOH). Amino acid ratios in 4 N methanesulfonic acid[18] hydrolysate: Asp 1.08, Thr 1.88, Pro 3.08, Gly 5.19, Ala 2.13, Val 3.00, Met 2.05, Leu 3.00, Tyr 0.98, Lys 1.07, His 1.98, Trp 0.85, Arg 1.00 (recovery of Leu, 82%). Amino acid ratios in a leucine-aminopeptidase digest (numbers in parentheses are theoretical): Thr 2.16 (2), Pro 2.93 (3), Gly 4.88 (5), Ala 2.08 (2), Val 2.92 (3), Met 1.95 (2), Leu 3.00 (3), Tyr 1.03 (1), Lys 1.12 (1), His 1.78 (2), Trp 0.88 (1), Arg 1.00 (1), Asn not determined (recovery of Leu, 77%).

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An Improved Synthesis of N^4-Aminocytidine

KAZUO NEGISHI, MASUMI KAWAKAMI, KIKUKO KAYASUGA, JUNICHI ODO, and HIKOYA HAYATSU*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

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N^4-Aminocytidine, a mutagenic nucleoside, was obtained as crystals on reaction of cytidine with a reagent mixture consisting of hydrazine, bisulfite and phosphate buffer at pH 7. The product can be precipitated directly from the reaction mixture, and therefore the procedure is suitable for preparing N^4-aminocytidine in large quantities.

Keywords——N^4-aminocytidine; large-scale synthesis; hydrazine; bisulfite; nucleoside analog; base-pair change; mutagen; transamination; cytidine

N^4-Aminocytidine (**1** in Fig. 1) and its N'-alkyl derivatives are potent mutagens in bacteria[1,2] and phages.[1-3] N^4-Aminocytidine is also mutagenic in cultured mammalian cells[4] and in *Drosophila*.[5] This nucleoside analog causes base-pair transitions, AT to GC and GC to AT, as shown by deoxyribonucleic acid (DNA) sequence determination of the mutant genes derived from phages ϕX174 *am*3[3] and M13 (unpublished work). When Chinese hamster V79 cells grow in the presence of N^4-aminocytidine in the culture medium, the cells incorporate this nucleobase analog into thier DNA.[4] N^4-Aminocytosine nucleotide can be incorporated into phage DNA *in vitro*, and the resulting DNA can induce the production of mutant phages in host bacteria.[6,7]

Fig. 1. Synthesis of N^4-Aminocytidine from Cytidine

In these studies, N^4-aminocytidine was prepared from cytidine by use of the bisulfite-mediated substitution of the N^4-amino group with hydrazine, which is a method originally used by Budowsky and coworkers for preparing N^4-aminodeoxycytidine 5′-phosphate from deoxycytidine 5′-phosphate.[8] N^4-Aminocytidine was obtained as a glassy material after fractionation of the reaction mixture by high-performance liquid chromatography (HPLC). Due to the necessity of the HPLC fractionation, the preparation has been limited to sub-gram scale. To extend the studies on the biological properties of N^4-aminocytidine, it is desirable to improve the method of isolation. Here we report the preparation of this compound in a crystalline form and describe a simple procedure to obtain the crystals in gram quantities.

Experimental Procedures

Synthesis and Purification of N^4-Aminocytidine——A reagent mixture was prepared by mixing hydrazine hydrate (4 ml, 80 mmol; Wako Pure Chemicals, Osaka), sodium bisulfite (0.2 g, 2 mmol; Wako), sodium dihydrogen phosphate dihydrate (0.3 g, 1.9 mmol; Wako) and water (10 ml). This mixture was neutralized to pH 7.0 by addition of concentrated HCl, and the volume was made up to 20 ml by adding water. Cytidine (0.3 g, 1.2 mmol) was dissolved in the reagent mixture (3 ml), and the resulting solution was allowed to stand at 60 °C for 4 h. The reaction mixture was then subjected to a preparative HPLC on a reversed phase column (ODS-20, i.d. 22 mm × 300 mm; Kusano Scientific Instruments, Tokyo) which had been connected to a precolumn (ODS-G3, i.d. 22 mm × 100 mm; Wako). After injection of the total reaction mixture into the column, elution was done with 50 mM formic acid, the pH of which had been adjusted to 4.5 by addition of ammonia. The flow rate was 3 ml/min. Ultraviolet (UV)-absorbing fractions ($A_{280} > 5$) were collected (t_R, 30—45 min) and evaporated to dryness under reduced pressure. The residue obtained was dissolved in water (2 ml) and rechromatographed on the same columns using water as the eluent. Fractions containing the product were combined and evaporated to dryness to give a glassy material (0.18 g). On storage in a freezer for several days, this material solidified. The solid was crystallized from water–ethanol to give colorless needles.

Gram-Scale Preparation of Crystalline N^4-Aminocytidine——Hydrazine hydrate (10 ml, 200 mmol), sodium bisulfite (0.5 g, 5 mmol), and sodium dihydrogen phosphate dihydrate (8 g, 32 mmol) were dissolved in water (30 ml). This solution was adjusted to pH 7.3 by addition of concentrated HCl (about 11 ml). Cytidine (15 g, 62 mmol) was added and the resulting solution was heated at 62 °C for 4.5 h. The solution was cooled in ice and a small amount of crystalline N^4-aminocytidine was added to it as seeds for the crystallization. Scratching with a spatula on the inside wall of the glass container facilitated the formation of crystals. After standing overnight at 4 °C, the crystals were collected by filtration, and washed with cold water (5 ml × 2), cold 50% methanol (30 ml × 2), methanol (50 ml × 2), and diethyl ether (50 ml × 3), successively. This crude sample (6 g) was recrystallized from water (16 ml) to give colorless crystals of N^4-aminocytidine hemihydrate (4.8 g; 29% of the theoretical yield). When this sample was analyzed by HPLC, it was 99.5% pure in terms of UV-absorbing (at 254 nm) material, the contaminating material being cytidine. For determination of the physical properties of the compound, the material was further recrystallized from water.

Care must be taken to avoid direct contact with this compound, because it is a potent mutagen.

Spectra——UV spectra were recorded on a Hewlett Packard 8450A spectrophotometer, nuclear magnetic resonance (NMR) spectra on a Hitachi R-22FT NMR spectrometer at 90 MHz with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as an internal standard, and Raman spectra on a JASCO NR-1000 spectrophotometer, with Ar-laser excitation at 514.5 nm (NEC 3300).

Results and Discussion

By a simple one-step reaction, gram quantities of N^4-aminocytidine were prepared from cytidine. In this procedure, the reaction mixture contains a high concentraion of the nucleoside, and the product can be precipitated directly from the mixture as a crystalline solid. The reaction solution also contains a high concentration of sodium phosphate, which serves as a catalyst to remove bisulfite from the 5,6-dihydropyrimidine 6-sulfonate intermediate.[9] The results of elemental analysis of the material indicated that it was a hemihydrate of N^4-aminocytidine: $C_9H_{14}N_4O_5 \cdot 1/2H_2O$ requires C, 40.45%; H, 5.66%; N, 20.97%; the values found were C, 40.20%; H, 5.60%; N, 20.82%. The mass spectrum (MS) gave a signal at m/z: 258 (the molecular weight of N^4-aminocytidine is 258.24). The UV spectra of N^4-aminocytidine at several pH values are shown in Figure 2: at pH 1, λ_{max} 281 nm (ε, 13700); at pH 7, λ_{max} 236 nm (ε, 7700) and 273 nm (ε, 10900); at pH 13, λ_{max} 265 nm (ε, 12600). The ε value at the λ_{max} at pH 7 is identical as that reported earlier for N^4-aminodeoxycytidine 5'-phosphate, which was determined on the basis of phosphorus analysis.[1] The spectra at pH 7 and 1 are very similar to those of cytidine at these pH values. The spectrum at pH 13, however, is very different from that of cytidine. The spectrum of N^4-aminocytidine at pH 13 is not that of some degradation products, because the spectrum reverted to that at pH 7 on neutralization of the alkaline solution. Probably, the hydrogen at the N^4 position dissociates under alkaline conditions. Titration of an acidic solution of N^4-aminocytidine with sodium hydroxide showed that this compound has a pK_a value of 4.50. In this titration, consumption

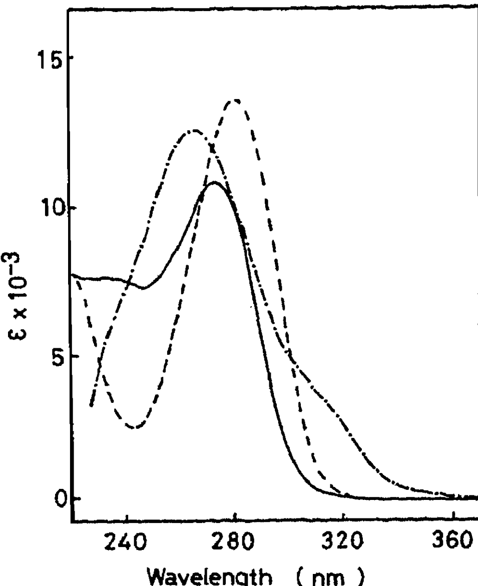

Fig. 2. UV Spectra of N^4-Aminocytidine

Spectra of N^4-aminocytidine solutions at various pH values.
pH 1, ----; pH 7, ——; pH 13, —·—.

of alkali was also observed at the pH region of 11—13, but the end point of the consumption was obscure. This poorly defined end point was apparently due to the dissociation of the sugar hydroxyl groups which occurs under strongly alkaline conditions. The dissociation of the N^4-hydrogen probably has its pK_a value at around 12.

In the Raman spectrum of solid N^4-aminocytidine, ν-NH_2 signals were observable at $3200\,cm^{-1}$ and $3313\,cm^{-1}$. Under the same conditions, cytidine gave ν-NH_2 signals at $3300\,cm^{-1}$ and $3452\,cm^{-1}$. The location of the signals for N^4-aminocytidine at smaller wave numbers than those for cytidine is consistent with the structure of the compound, in which the NH_2 is not directly linked to the pyrimidine ring.

Proton nuclear magnetic resonance (^1H-NMR) spectra recorded in CF_3COOD–D_2O (3:10, v/v) gave signals at δ5.90 ppm (1H, d, J=3.5 Hz) for 1′-H, δ6.25 ppm (1H, d, J= 7.5 Hz) for 5-H, δ8.14 ppm (1H, d, J=7.5 Hz) for 6-H, and complex signals in the 4 ppm region due to protons in the sugar moiety.

The preparation of N^4-aminocytosine nucleoside derivatives has been described.[8,10-12] However, N^4-aminocytidine is not among those reported. The method of preparation described in the earlier literature[10-12] involved the substitution of 4-thio- or 4-alkylthiouracil (or thymine) nucleosides with hydrazine. Obviously, the bisulfite-mediated amine exchange of cytosine nucleosides[8] is a simpler way of preparing N^4-aminocytosines. An advantage of this method is that the reaction takes place in neutral aqueous solution, so that it can be adapted to the preparation of N^4-aminocytosine nucleotides and polynucleotides.

The availability of large quantities of crystalline N^4-aminocytidine will facilitate its use in chemical and biological studies. For example, a study on metal complexes of N^4-aminocytidine is in progress, and a test for carcinogenic potential of N^4-aminocytidine in animals is also under way.

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Pharmacological Activities of Glycyrrhetinic Acid Derivatives: Analgesic and Anti-Type IV Allergic Effects

HIDEO INOUE,*[,a] TAKEO MORI,[a] SHOJI SHIBATA,[b] and HIROSHI SAITO[c]

Research Laboratory, Minophagen Pharmaceutical Co.,[a] 2–5233, Komatsubara, Zama-shi, Kanagawa 228, Japan, Laboratory of Natural Medicinal Materials, Minophagen Pharmaceutical Co.,[b] 3–2–7, Yotsuya, Shinjuku-ku, Tokyo 160, Japan and Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo,[c] Hongo, Bunkyo-ku, Tokyo 113, Japan

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Glycyrrhetinic acid (Ia) and fourteen derivatives of glycyrrhetinic acid were examined to estimate the antinociceptive activity (tests on writhing and vascular permeability induced by 0.7% acetic acid) and anti-type IV allergic effects in mice. Among them, three dihemiphthalate compounds (IIf, IIIc, IVc) derived from, deoxoglycyrrhetol (IIa), showed strong inhibition of writhing and dye leakage at a dose of 6.25 mg/kg (*p.o.*) and type IV allergy at 25 mg/kg (i.p.). Among the hemisuccinate compounds, Ib, IIb and IIIb, had significant inhibitory effects on both writhing and vascular permeability, whereas IIc and IVb inhibited only one of the two responses, at 200 mg/kg (*p.o.*). Four of the hemisuccinate compounds (IIb, c, IIIb, IVb) suppressed type IV allergy at 100 mg/kg (i.p.). Glycyrrhetinic acid (Ia) and deoxoglycyrrhetol (IIa) showed no detectable inhibition in the screening tests at doses of less than 200 mg/kg (i.p.).

Keywords——glycyrrhetinic acid; antinociceptive activity; type IV allergy; dihemiphthalate compound; hemisuccinate compound; deoxoglycyrrhetol; oleanane skeleton

Introduction

Glycyrrhetinic acid (Ia), the aglycone of glycyrrhizin, which is obtained from the root of licorice (*Glycyrrhiza* spp.), is an effective anti-inflammatory agent.[1] Nishino *et al.*[2] found an inhibitory effect of glycyrrhetinic acid on tumor-promoting activity. Glycyrrhetinic acid has recently been shown to be an inhibitor of intercellular junctional communication.[3] Carbenoxolone sodium (Ib) prepared from glycyrrhetinic acid, is clinically employed in the therapy of gastric ulcer.[4] A glycyrrhetinic acid derivative, the sodium salt of glycyrrhetinic acid hemiphthalate (Ic), was demonstrated to have an anti-inflammatory effect.[5] It has also been reported that deoxoglycyrrhetol (IIa) inhibits experimental gastric ulcer and allergy.[6] Furthermore, three dihemiphthalate compounds (the disodium salts of 18β-olean-12-ene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IIf), 18β-olean-9(11),12-diene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IIIc), and olean-11,13(18)-diene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IVc)) have been shown to prevent gastric ulcer.[7] Previously we reported that among fifteen derivatives of glycrrhetinic acid so far tested, the dihemiphthalate compounds (IIf, IIIc and IVc) showed strong inhibitory effects on the 5- and 12-lipoxygenase and cyclooxygenase activities in cloned mastocytoma.[8]

The present paper describes a further pharmacological study on these compounds in order to examine whether some of them are effective in the writhing and type IV allergy tests.

Ia : R=H, R'=H
Ib : R=NaOOC(CH_2)$_2$CO-, R'=Na
Ic : R= -OC(C$_6$H$_4$)COONa , R'=Na

IIa : R=H, R'=H
IIb : R=NaOOC(CH_2)$_2$CO-, R'=H
IIc : R=NaOOC(CH_2)$_2$CO-, R'=NaOOC(CH_2)$_2$CO-
IId : R=-P(=O)(ONa)ONa , R'=-P(=O)(ONa)ONa
IIe : R=-OC(C$_6$H$_4$)COONa , R'=H
IIf : R=-OC(C$_6$H$_4$)COONa , R'=-OC(C$_6$H$_4$)COONa

IIIa : R=H, R'=H
IIIb : R=NaOOC(CH_2)$_2$CO-, R'=H
IIIc : R=-OC(C$_6$H$_4$)COONa , R'=-OC(C$_6$H$_4$)COONa

IVa : R=H, R'=H
IVb : R=NaOOC(CH_2)$_2$CO-, R'=H
IVc : R=-OC(C$_6$H$_4$)COONa , R'=-OC(C$_6$H$_4$)COONa

Chart 1

Materials and Methods

Animals——Male ddY mice (6—7 weeks old) used in this experiment were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Animals were housed in an air-conditioned room at 22±1 °C with 55±5% relative humidity, and were given free access to food and water. They were used after taming for 1 week.

Test Compounds——Glycyrrhetinic acid, its derivatives and phthalic acid were prepared by Mr. N. Nagata, Minophagen Research Laboratory. Aminopyrine (Sigma Chemical Co., U.S.A.) for the writhing tests and prednisolone (Shionogi Pharmaceutical Co., Japan) for type IV allergy were used as positive controls.

Assay for Analgesic Activity——Analgesic effects of the compounds were estimated in terms of the effects on writhing and vascular prermeability induced by acetic acid. The test compounds were dissolved in physical saline containing 1% Tween 80 and given orally 45 min prior to the intraperitoneal injection (10 ml/kg) of 0.7% acetic acid solution; 0.1 ml/animal of 4% Evans blue was also intravenously given at 10 min before the administration of acetic acid. The number of writhes of each mouse was counted during a period of 20 min after the acetic acid injection. For the examination of vascular permeability, the mice used in the writhing tests were immediately sacrificed and injected with 3 ml of saline intraperitoneally. The fluid collected from the peritoneal cavity was added to 2 ml of saline and centrifuged at 3000 rpm for 10 min. The dye in the supernatant was measured spectrophotometrically at 590 nm. The results were expressed as inhibition (percent) of writhing and dye-leakage in the test groups as compared with the control.

Assay for Anti-Allergic Activity——The anti-allergic effect of the compounds was examined on picryl chloride (PC)-induced type IV allergy in the ear of mice. Sensitization of mice was performed according to the method of Asherson and Ptak.[9] Firstly, the abdomen of mice (shaved prior to the experiment) was treated with 100 μl of 7% PC-ethanol solution. Seven days after PC-treatment, the animals were challenged with 20 μl/ear of antigen (1% PC-olive oil) on both ears, and after further 5 d, these sensitization were repeated. The test compounds, dissolved in physical saline containing 1% Tween 80, were intraperitoneally injected into mice at 0, 3 and 9 h after the second challenge. The ear thickness was measured with a dial thickness guage (Ozaki Factory, Japan) with the unit of 0.01 mm at 9 and 24 h after the second challenge. The results were expressed as the inhibition (percent) of swelling relative to the control, after subtracting the thickness before challenge.

Data Analysis——Student's *t*-test was used to determine statistical significance.

Results and Discussion

Effects of Glycyrrhetinic Acid Derivatives on Writhing and Vascular Permeability

The effects of the compounds tested on the acetic acid-induced writhing and vascular permeability are summarized in Table I. In the control groups, the number of writhes was 26.7 ± 1.4 (S.E.) while the OD value of peritoneal dye-leakage was 0.403 ± 0.03 (S.E.) under our experimental conditions. Some of the test compounds inhibited both writhing and vascular permeability. Three hemiphthalate compounds [the disodium salts of 18β-olean-12-ene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IIf), 18β-olean-9(11), 12-diene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IIIc) and olean-11,13(18)-diene-3β,30-diol 3β,30-di-*O*-hemiphthalate (IVc)] exhibited the strongest inhibition of writhing and vascular permeability, and showed significant effects at a dose of 6.25 mg/kg (*p.o.*). The hemisuccinate compounds (IIb, c and

TABLE I. Inhibition of Acetic Acid-Induced Writhing and Vascular Permeability by Glycyrrhetinic Acid Derivatives

Compound	Dose (mg/kg) (*p.o.*)	Writhing	Dye-leakage
Ia	200	13	13
Ib	100	3	28
	200	58[a]	43[b]
Ic	100	15	14
	200	18	0
IIa	100	21	11
	200	24	22
IIb	100	0	7
	200	66[a]	32[b]
IIc	100	7	23
	200	45[b]	26
IId	100	0	28
	200	28	29[b]
IIe	100	18	18
	200	36	38[b]
IIf	3.13	20	19
	6.25	32[b]	34[b]
	12.5	29[b]	58[a]
	25	43[b]	55[a]
IIIa	200	13	11
IIIb	100	22	25
	200	40[b]	37[b]
IIIc	3.13	21	9
	6.25	45[b]	50[b]
	12.5	37[b]	39[b]
	25	64[b]	55[a]
IVa	200	29	19
IVb	100	6	0
	200	35	39[b]
IVc	3.13	22	17
	6.25	46[b]	59[b]
	12.5	71[a]	48[a]
	25	63[a]	49[a]
Phthalic acid	200	16	0
Aminopyrine	200	100[c]	70[a]

Values of inhibition are expressed as percent of the control ($n=7$—8). *a*) $p<0.01$; *b*) $p<0.05$; *c*) $p<0.001$.

IIIb) including carbenoxolone sodium (Ib), which have a sodium succinate group at position 3 or 30 in the oleanane skeleton, showed activity at 200 mg/kg (*p.o.*), but a dose of less than 100 mg/kg (*p.o.*) exhibited no apparent inhibition. In contrast, glycyrrhetinic acid (Ia), deoxoglycyrrhetol (IIa), 18β-olean-9(11), 12-diene-(IIIa) and 18β-olean-11,13(18)-diene-3β,30-diol (IVa), the skeletal compounds of the above derivatives, were found to have weak inhibitory effects on the writhing response. The compounds having sodium hemiphthalate groups at the 3- and 30- positions of rings A and E in the oleanane skeleton (IIf, IIIc and IVc) showed potent inhibition. The monosodium salt of 18β-olean-12-ene-3β,30-diol 3β-*O*-hemiphthalate (IIe) showed a slight effect, but it was not statistically significant. Compound Ic was less effective in inhibiting writhing than glycyrretinic acid (Ia). In addition, we confirmed that phthalic acid itself did not affect writhing or vascular permeability induced by acetic acid. These results suggest that dihemiphthalate substitution on the oleanane skeleton is required for a potent inhibitory effect on writhing and vascular permeability.

Effect of Glycyrrhetinic Acid Derivatives on Type IV Allergy

A primary screening of glycyrrhetinic acid derivatives was performed for inhibitory effect on PC-induced type IV allergy (Table II). The compounds tested were given intraperitoneally immediately after the second challenge. Each value was expressed as inhibition (percent) of ear swelling at 9 h after challenge. Some compounds (Ib, c, IIe, f, IIIc and IVc) were administered at less than 50 mg/kg since their LD_{50} (i.p.) values were 60 to 150 mg/kg. As shown in Table II, three dihemiphthalates (IIf, IIIc and IVc) and four hemisuccinates (IIb, c, IIIb and IVb), showed significant inhibition of type IV allergy at 25 to 100 mg/kg. Another hemisuccinate compound, carbenoxolone sodium (Ib), was also found to have inhibitory activity, but Ia, IIa, IIIa and IVa did not have significant effects. The inhibitory effects of the seven derivatives (IIb, c, f, IIIb, c, IVb, c) were further investigated (Table III). These compounds showed potent inhibitory activity, suppressing about 60% of ear swelling when

TABLE II. Inhibition of PC-Induced Contact Dermatitis by Glycyrrhetinic Acid Derivatives

Compound	Dose (mg/kg)	Inhibition (%)	Compound	Dose (mg/kg)	Inhibition (%)
Ia	100	15	IIIa	200	6
	200	35	IIIb	50	18
Ib	25	14		100	32[a]
	50	22[a]		200	43[b]
Ic	50	0	IIIc	12.5	4
IIa	100	15		25	42[a]
	200	18	IVa	100	21
IIb	50	0		200	0
	100	23[a]	IVb	50	0
	200	33[b]		100	44[a]
IIc	50	0		200	43[b]
	100	55[b]	IVc	12.5	20
	200	76[b]		25	45[b]
IId	100	17	Prednisolone	5	42[b]
	200	52[b]		10	77[c]
IIe	25	16			
IIf	12.5	13			
	25	19[a]			

Values of inhibition are expressed as percent of the control at 9 h after challenge ($n=7$—8). Compounds were given i.p. immediately after challenge. *a*) $p<0.05$; *b*) $p<0.01$; *c*) $p<0.001$.

TABLE III. Inhibition of PC-Induced Contact Dermatitis by Seven Derivatives of Glycyrrhetinic Acid

Compound	Dose (mg/kg)	Inhibition (%) after challenge	
		9 h	24 h
IIb	100	33	36
	200	57[a]	54[b]
IIc	50	16	34
	100	58[a]	69[a]
	200	53	85[b]
IIf	12.5	23	22
	25	59[b]	66[b]
IIIb	100	44	35
	200	58[b]	70[b]
IIIc	12.5	41	28
	25	58[b]	61[b]
IVb	50	39	25
	100	61[b]	64[b]
	200	58[b]	74[b]
IVc	12.5	41	23
	25	64[b]	70[b]
Prednisolone	5	60[b]	46[b]

Values of inhibition are expressed as percent of the control (n=7—8). Compounds were given i.p. at 3 and 9 h after challenge. *a*) $p<0.05$; *b*) $p<0.01$.

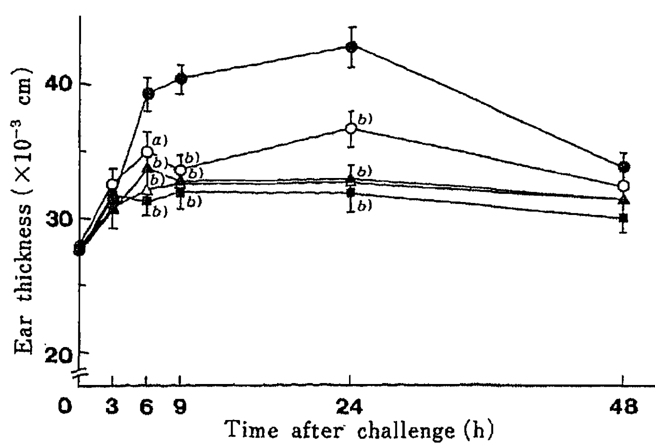

Fig. 1. Inhibitory Effect of Dihemiphthalate Compounds on Type IV Allergy

Each value is the mean±S.E. for 8 animals. Test compounds were given i.p. at 3 and 9 h after challenge. The assay was carried out as described in Materials and Methods.
(●), saline; (○), prednisolone 5 mg/kg; (△), compound IIf 25 mg/kg; (▲), compound IIIc 25 mg/kg; (■), compound IVc 25 mg/kg. *a*) $p<0.05$; *b*) $p<0.01$.

administered twice at 3 and 9 h after challenged. In particular, the inhibitory effects at 24 h after challenge were more potent than those of prednisolone (5 mg/kg). The dihemiphthalate compounds (IIf, IIIc and IVc) were found to inhibit the ear swelling significantly at 6 h after challenge (Fig. 1). It is a characteristic of those compounds that the time-course of the ear swelling treated with them was similar to that in the case of prednisolone. However, we do not yet have enough information to discuss the mechanism of inhibitory effect on PC-induced type IV allergy.

We confirmed that some of the glycyrrhetinic acid derivatives inhibited writhing and/or

peritoneal dye-leakage, as well as type IV allergy. In particular, the dihemiphthalate compounds (IIf, IIIc and IVc) strongly inhibited both writhing and peritoneal leakage, suggesting that these compounds have anti-inflammatory and antinociceptive activity. Previously we found that the dihemiphthalate compounds (IIf, IIIc and IVc) strongly inhibit lipoxygenase and cyclo-oxygenase activities,[8] and prevent the formation of gastric ulcer.[7] Therefore, these compounds are candidates as drugs to treat inflammation and allergic disease without unfavorable gastric side effects.[7]

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5-Fluorouracil Derivatives. XII.[1] Synthesis and Antitumor Activity of α-Alkylthiomethyl-, α-Alkylsulfinylmethyl-, α-Alkylsulfonylmethyl-, and α-Acylthiomethyl-5-fluorouracils

SHOICHIRO OZAKI,*,[a] TOSHIO NAGASE,[a] HIROFUMI TAMAI,[a] HARUKI MORI,[b] AKIO HOSHI,[c] and MASAAKI IIGO[c]

Faculty of Engineering, Ehime University,[a] Bunkyo-cho, Matsuyama 790, Japan, Research Center. Mitsui Toatsu Chemicals,[b] Totsuka-ku, Yokohama 247, Japan, and National Cancer Center Research Institute,[c] Chuo-ku, Tokyo 104, Japan

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For the purpose of diminishing the toxicity of 5-fluorouracil (**1**) and obtaining biologically active derivatives of **1** suitable for oral administration, alkylthiomethyl, alkylsulfinylmethyl, alkylsulfonylmethyl, and acylthiomethyl groups were introduced at the 1- and 3-positions of **1**. The antitumor activity of these synthetic compounds was tested against L1210 leukemia in mice. 1-Alkylthiomethyl-5-fluorouracils showed weak antitumor activity at a high dose (300 mg/kg).

Keywords——5-fluorouracil; α-alkylthiomethyl-5-fluorouracil; α-alkylsulfinylmethyl-5-fluorouracil; α-alkylsufonylmethyl-5-fluorouracil; antitumor agent

During various synthetic studies on masked 5-fluorouracil derivatives, we have realized that the bond strength between the nitrogen atom in the uracil ring and the substituent X is an important factor influencing the antitumor activity and the toxicity of the compounds. The previous results indicated that the weaker the bond strength, the stronger the antitumor activity and the toxicity of the masked compounds. In the case of *N*-carbonyl-5-fluorouracil derivatives,[2,3] for example, the N–C bond was relatively labile and its lability resulted in more potent antitumor activities as well as undesired side-effects. In the case of *N*-alkyl-5-fluorouracil derivatives,[4,5] the strong N–C bond conversely prevented these derivatives from being easily hydrolyzed *in vivo* and from showing any antitumor activity against L1210 leukemia. When oxygen was introduced at the α-position to the alkyl group, the N–C bond became labile under hydrolytic conditions and the resulting 1-acyloxyalkyl-,[6] 1-alkoxyalkyl-,[7,8] and 1-(tetrahydro-2-furyl)-5-fluorouracils[2] showed moderate antitumor activity. In view of these results, the authors thought that if sulfur instead of oxygen were introduced at the α-position to the alkyl substituent, this perturbation might modify the antitumor activity. In this report we wish to present details of the synthesis of α-alkylthiomethyl, α-alkylsulfinylmethyl-, α-alkylsulfonylmethyl-, and acylthiomethyl-5-fluorouracil derivatives[9] and their antitumor activities.

Introduction of sulfur-containing substituents into the 1- and/or 3-position(s) of 5-fluorouracil was effected in two different ways. In the first process, 2,4-bis(trimethylsiloxy)-5-fluoropyrimidine was chosen as the starting material and treated with alkylthiomethyl

X: alkyl, $-\overset{O}{\overset{\|}{C}}R$, $-CH_2OR$, $-CH_2SO_nR$ ($n=0, 1, 2$)

Fig. 1

2a: R=Me
2b: R=Et

Chart 1

a: R = Me
b: R = Et

Chart 2

5: X=H
6: X=CH_2Cl

7: X=H
8: X=CH_2SCOR
a: R=Me
b: R=Ph

Chart 3

TABLE I. Antitumor Activity of 5-Fluorouracil Derivatives in the L1210 Leukemia

Compound No.	Substituent	Dose[a] (mg/kg/d)	ILS[b] (%)
2a	1-CH_2SCH_3	i.p. 100	−4
		p.o. 100	−6
		p.o. 300	11
2b	1-$CH_2SC_2H_5$	i.p. 30	−1
		i.p. 100	−4
		p.o. 100	−2
		p.o. 300	8
3a	1-CH_2SOCH_3	i.p. 100	−8
		p.o. 100	3
3b	1-$CH_2SOC_2H_5$	i.p. 30	−10
		i.p. 100	−5
		p.o. 100	−2
		p.o. 300	−8
4a	1-$CH_2SO_2CH_3$	*p.o.* 100	2
4b	1-$CH_2SO_2C_2H_5$	*p.o.* 100	0
7a	3-CH_2SCOCH_3	*p.o.* 100	4
7b	3-$CH_2SCOC_6H_5$	*p.o.* 100	0
8a	1,3-CH_2SCOCH_3	*p.o.* 100	−9

a) i.p. and *p.o.* mean intraperitoneal and *per os* administration, respectively. *b*) ILS means increase in life span; see Experimental.

chlorides in the presence of stannic chloride to afford 1-alkylthiomethyl-5-fluorouracils (**2**) after usual work-up (Chart 1). In this step, no 3-alkylthiomethyl derivatives were isolated. Both 1-alkylsulfinylmethyl- and 1-alkylsulfonylmethyl-5-fluorouracils (**3** and **4**) were easily prepared by the subsequent oxidation of **2** with sodium periodate. Compound **2** was converted to **3** by low-temperature oxidation (at 0 °C), and to **4** by high-temperature oxidation (at 90—100 °C). In both cases, the desired derivatives were obtained in good to excellent yields (Chart 2).

Acylthiomethyl-5-fluorouracils were prepared by direct esterification of thiocarboxylic acids with *N*-chloromethyl-5-fluorouracils **5** and **6**.[10] The reactions did not occur in the absence of triethylamine, but **5** and **6** were consumed within several hours even at low temperature in the presence of a base (Chart 3).

The antitumor activity of the nine-prepared compounds was tested against L1210 leukemia in mice,[11] and the results (increase in life span) are shown in Table I. 1-Alkylthiomethyl-5-fluorouracils **2** showed weak antitumor activity at a high dose (300 mg/kg) but compounds **3**, **4**, **7**, and **8** showed no antitumor activity. These data suggested that the perturbation caused by introducing the sulfur atom as the α-position to the alkyl group was not effective in weakening the N–C bond *in vivo*, compared with that caused by the presence of the oxygen atom, so that the sulfur-containing *N*-alkyl-5-fluorouracil derivatives unfortunately had little or no antitumor activity against L1210 leukemia.

Experimental

The melting points were recorded on a Büchi melting point apparatus and are uncorrected. The infrared (IR) spectra were obtained on a JASCO IR-A-1 spectrometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on JEOL-60HL, JEOL FX-100, and Hitachi R-24 spectrometers.

1-Methylthiomethyl-5-fluorouracil (2a)——Stannic chloride (5.21 g, 0.02 mol) was added dropwise to a mixture of 2,4-bis(trimethylsiloxy)-5-fluoropyrimidine (13.72 g, 0.05 mol), chloromethyl methyl sulfide (4.83 g, 0.05 mol), and chloroform (10 ml) at 60 °C. After stirring of the mixture for 1 h, ethanol (19 ml) was added and the whole was evaporated. The residue was taken up in dichloromethane (200 ml), and this solution was washed with water, dried, and evaporated. Ether was added to the residual oil to solidify the product. Filtration of this mixture gave **2a** (4.87 g, 52%). mp 147—148 °C. IR (KBr): 3180, 3030, 2840, 1775, 1735, 1690, 1670, 1488, 1432, 1381, 1350, 1295, 1250, 1234, 1127, 1003, 756, 711 cm^{-1}. ^{1}H-NMR (CD_3COCD_3) δ: 2.23 (3H, s, CH_3), 4.88 (2H, s, CH_2), 7.78 (1H, d, J=6 Hz, C_6-H), 10.50 (1H, br, N_3-H). *Anal.* Calcd for $C_6H_7FN_2O_2S$: C, 37.89; H, 3.71; F, 9.99; N, 14.73. Found: C, 37.69; H, 3.70; F, 9.73; N, 14.66.

1-Ethylthiomethyl-5-fluorouracil (2b)——Compound **2b** was prepared from 2,4-bis(trimethylsiloxy)-5-fluoropyrimidine (5.49 g, 0.02 mol) and ethylthiomethyl chloride (2.21 g, 0.02 mol) in the presence of stannic chloride (5.21 g, 0.02 mol) at 70 °C. **2b** (1.7 g, 42%). mp 148—149 °C. IR (KBr): 3190, 3070, 2860, 1730 (C=O), 1675, 1430, 1382, 1360, 1252, 1241, 1134, 928, 771, 704 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=8 Hz, CH_3), 2.64 (2H, q, J=8 Hz, CH_2), 4.86 (2H, s, CH_2S), 7.53 (1H, d, J=6 Hz, C_6-H), 9.92 (1H, br, N_3-H). *Anal.* Calcd for $C_7H_9FN_2O_2S$: C, 41.17; H, 4.44; F, 9.30; N, 13.72. Found: C, 40.87; H, 4.20; F, 9.10; N, 13.48.

1-Methylsulfinylmethyl-5-fluorouracil (3a)——Compound **2a** (2.85 g, 0.015 mol) was added to a cooled solution of $NaIO_4$ (3.37 g, 0.0158 mol) in water (40 ml) at 0 °C, and this mixture was stirred for 30 min, then filtered. The filtrate was evaporated to dryness. The residue was dissolved in acetone and the insoluble materials were filtered off. This filtrate was evaporated to dryness, acetone was again added, the insoluble materials were filtered off, and the filtrate was evaporated to dryness. The residue was washed with ethanol to afford **3a** (2.07 g, 67%) as colorless crystals. mp 200—201 °C. IR (KBr): 3190, 3030, 2915, 2858 1727, 1706, 1672, 1489, 1418, 1390, 1365, 1252, 1146 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.20 (3H, s, CH_3), 4.67 (1H, d, J=13 Hz, CH), 5.00 (1H, d, J=13 Hz, CH), 7.93 (1H, d, C_6-H, J=6 Hz), 11.97 (1H, br, N_3-H). *Anal.* Calcd for $C_6H_7FN_2O_3S$: C, 34.95; H, 3.42; F, 9.21; N, 13.59; S, 15.55. Found: C, 34.83; H, 3.07; F, 8.96; N, 13.29; S, 15.25.

1-Ethylsulfinylmethyl-5-fluorouracil (3b)——In the same manner as used for the preparation of **3a**, **3b** (3.71 g, 84%) was obtained from **2b** (4.08 g, 0.02 mol) and $NaIO_4$ (4.49 g, 0.022 mol). mp 204 °C. IR (KBr): 3180, 1720, 1704, 1670, 1486, 1384, 1067 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.25 (3H, t, J=8 Hz, CH_3), 2.80 (2H, dq, J=8, 13 Hz, CH_2), 4.69 (1H, d, J=13 Hz, CH), 7.98 (1H, d, J=7 Hz, C_6-H), 12.02 (1H, br, N_3-H). *Anal.* Calcd for $C_7H_9FN_2O_3S$: C, 38.18; H, 4.12; F, 8.63; N, 12.72; S, 14.56. Found: C, 38.46; H, 4.05; F, 8.45; N, 12.44; S, 14.38.

1-Methylsulfonylmethyl-5-fluorouracil (4a)——Compound **2a** was added to a solution of $NaIO_4$ (4.91 g, 0.022 mol) in water (50 ml) with stirring at 90—100 °C. Compound **2a** soon dissolved and after 30 min, new crystals

deposited from the solution. Stirring was continued for an additional 1.5 h, then the solution was cooled in an ice bath and the precipitate was filtered off, washed with water, and dried to obtain **4a** (2.09 g, 91%). mp 248 °C (dec.). IR (KBr): 3200, 3080, 1732, 1708, 1675, 1475, 1389 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 3.06 (3H, s, CH_3), 5.14 (2H, s, CH_2), 8.03 (1H, d, C_6-H), 12.04 (1H, br, N_3-H). *Anal.* Calcd for $C_6H_9FN_2O_4S$: C, 32.43; H, 3.18; F, 8.53; N, 12.61; S, 14.43. Found: C, 32.73; H, 2.99; F, 8.44; N, 12.32; S, 14.51.

1-Ethylsulfonylmethyl-5-fluorouracil (4b)——Compound **4b** (0.87 g, 89%) was obtained by oxidation of **3b** (0.85 g, 0.0042 mol) with $NaIO_4$ (1.96 g, 0.009 mol) in the same manner as described for **4a**. mp 240—241.5 °C. IR (KBr): 3200, 1752, 1715, 1677, 1475, 1231, 1131 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 1.24 (3H, t, J=8 Hz, CH_3), 3.12 (2H, q, J=8 Hz, CH_2), 5.12 (2H, s, NCH_2), 8.03 (1H, d, J=6 Hz, C_6-H), 12.06 (1H, br, N_3-H). *Anal.* Calcd for $C_7H_9FN_2O_4S$: C, 35.59; H, 3.84; F, 8.04; N, 11.86; S, 13.57. Found: C, 35.74; H, 3.84; F, 8.34; N, 11.56; S, 13.86.

3-Acetylthiomethyl-5-fluorouracil (7a)——Triethylamine (0.105 ml, 0.75 mmol) was added to a mixture of 3-chloromethyl-5-fluorouracil (5)[10] (134 mg, 0.75 mmol) and thioacetic acid (74.3 mg, 0.975 mmol) in acetonitrile (10 ml) over 5 min at room temperature. Exothermic reaction occurred to produce a precipitate of triethylamine hydrochloride. After a few hours, the precipitate was filtered off and the filtrate was evaporaed to dryness. The residue was subjected to silica gel column chromatography (hexane: ethyl acetate = 1 : 1) to give **7a** (128 mg, 78%). mp 136—137 °C. IR (KBr): 3340, 3080, 1670 (vs), 1472, 1415, 1244 (s), 1192, 1116, 990 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 2.34 (3H, s, CH_3), 5.38 (2H, s, CH_2), 7.87 (1H, t, J=6 Hz, C_6-H, became a doublet when D_2O was added), 11.25 (1H, br, N_1-H). *Anal.* Calcd for $C_6H_6FN_2O_3S$: C, 38.53; H, 3.23; N, 12.84. Found: C, 38.66; H, 3.04; N, 12.95.

3-Benzoylthiomethyl-5-fluorouracil (7b)——According to the same method as used for **7a**, **7b** (1.5 g, 54%) was obtained from 3-chloromethyl-5-fluorouracil (1.78 g, 0.01 mol) and thiobenzoic acid (1.79 g, 0.015 mol). mp 240—241 °C. IR (KBr): 3207, 3080, 1707, 1665 (vs), 1630, 1418, 1312, 1200 (s), 900 (s), 800, 760 cm^{-1}. ^1H-NMR (DMSO-d_6) δ: 5.54 (2H, s, CH_2), 7.5—8.0 (6H, m, C_6H_5 and C_6-H), 11.32 (1H, s, NH).

1,3-Bis(acetylthiomethyl)-5-fluorouracil (8a)——Compound **8a** was prepared from 1,3-bis(chloromethyl)-5-fluorouracil and 2 eq of thioacetic acid in the presence of 2 eq of triethylamine as described above for **7a** and **7b**. **8a** (0.52 g, 70%). mp 70 °C. ^1H-NMR ($CDCl_3$) δ: 2.13 (3H, s, CH_3 at 1-position), 2.37 (3H, s, CH_3 at 3-position), 5.45 (2H, s, CH_2 at 3-position), 5.68 (2H, s, CH_2 at 1-position), 7.66 (1H, d, J=6 Hz, C_6-H). *Anal.* Calcd for $C_{10}H_{11}FN_2O_4S_2$: C, 39.22; H, 3.62; F, 6.20; N, 9.14. Found: C, 38.95; H, 3.35; F, 5.90; N, 9.04.

Animals and Tumor System——Male BDF_1 mice weighing 20 ± 2 g were used. Six mice in each group, either test or control, were implanted intraperitoneally with 1×10^5 cells of L1210 leukemia. The compound to be tested was injected intraperitoneally or administered orally once daily for 5 d, starting 24 h after tumor implantation.

Evaluation of Antitumor Activity——The increase in life span was calculated by using the following formula:

$$\text{ILS (increase of life span) (\%)} = (T - C)/C \times 100$$

where T is the average number of days before death in the test group and C is the average number of days before death in the control group.[11]

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Synthesis and Antihypertensive Activities of 1,4-Dihydropyridine-5-phosphonate Derivatives. I[1]

IWAO MORITA,* SHIN-ICHI TADA, KATSUTOSHI KUNIMOTO, MASAMI TSUDA, MASAHIRO KISE, and KIYOSHI KIMURA

Research Laboratories, Nippon Shinyaku Co., Ltd., Nishiohji-Hachijo, Minami-ku, Kyoto 601, Japan

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A series of phosphonate derivatives, designed as analogues of 1,4-dihydropyridine-3,5-dicarboxylates in which a phosphonate group was introduced instead of the carboxylate group at the 5-position, was synthesized, and their antihypertensive activities were examined. Among the compounds examined, 5-diallyloxyphosphinyl-1,4-dihydropyridine-3-carboxylates were most effective in lowering the blood pressure in normotensive rats and spontaneously hypertensive rats (SHR). The phosphonate derivatives are considered to be close analogues of the carboxylate derivatives. The structure–activity relationships are discussed.

Keywords——1,4-dihydropyridine derivative; phosphonate derivative; calcium antagonist; antihypertensive activity; nifedipine; nicardipine; structure–activity relationship

Nifedipine[2] and nicardipine[3] are widely used clinically in the treatment of angina pectoris and hypertension, and a number of 1,4-dihydropyridine-3,5-dicarboxylate derivatives[4] have been reported to possess calcium-antagonistic activity. It has been generally recognized that the carboxylate moieties in these agents are essential for calcium-antagonistic activity.[5]

Our effort to find a new type of calcium antagonists was focused on replacing the usual carboxylate moiety with a phosphonate moiety, which was expected to be very hydrophilic, so that the bioavailability and/or pharmacokinetic parameters of the drugs coud be modulated. In this paper we describe the synthesis, pharmacological activities and structure–activity relationships of several 1,4-dihydropyridine-5-phosphonate derivatives. Although these compounds have been noted in the literature,[6] their biological activities have not been described.

Chemistry

The 1,4-dihydropyridine-5-phosphonate derivatives [I: 7—37] listed in Table I were synthesized *via* the routes shown in Chart 1. The preparation of compounds I (R^1,R^2 = Et; Ar = C_6H_5, 4-MeO–C_6H_5) has been described by Issleib *et al.*[6] In the same manner, the 1-

nifedipine nicardipine I

Fig. 1

$(R^1O)_3P$ **1** $\xrightarrow{BrCH_2COCH_3}$ $(R^1O)_2\overset{O}{\overset{\|}{P}}CH_2COCH_3$ **2** $\xrightarrow{\text{Ar-CHO } \mathbf{4}}$

$\longrightarrow$ $(HC{\equiv}CCH_2O)_2\overset{O}{\overset{\|}{P}}\text{-}CH{=}C{=}CH_2$ **3** $\xrightarrow{NH_4OH}$ **2**

$\text{Ar-CH}{=}\underset{COCH_3}{C}\text{-}\overset{O}{\overset{\|}{P}}(OR^1)_2$ **5** $\xrightarrow{CH_3\underset{NH_2}{C}{=}CHCO_2R^2\ \mathbf{6}}$ I

Chart 1

arylideneacetonylphosphonates **5** were allowed to react with the appropriate 3-aminocrotonates **6** in 2-propanol under reflux to afford 1,4-dihydropyridines (I) in 12—47% yields (method A). When compounds **5** were used without further purification (method B), the yields of (I) were low (2—35%, from **2**). The 1,4-dihydropyridines (I) were also prepared directly from the acetonylphosphonates **2**, the appropriate arylaldehydes **4** and **6** in the presence of piperidine–AcOH salt (method C).

In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum the values of the P–H coupling constants and the chemical shifts in the 1,4-dihydropyridines (I) were 4.7—5.1 (1H, d, $J=10$—11 Hz, C_4-H), 5.8—6.2 (1H, d, $J=5$—6 Hz, NH) and 2.25—2.35 (3H, d, $J=2$—3 Hz, C_6–CH_3), which clearly indicate the formation of the desired phosphonates.

The intermediates **5** were prepared by condensation of **2** and **4** in the presence of a catalyst in benzene by means of the Knoevenagel reaction, in 15—70% yield. The intermediates **2** and **6** were prepared as follows. The Arbuzov reaction[7] of the phosphites **1** with bromoacetone afforded **2** in 33—69% yields. However, the propargyl ester of **2** (not obtained under the Arbuzov reaction conditions) was prepared by reaction of ammonia with the allene intermediate[8] **3** obtained by the thermal rearrangement of tripropargyl phosphite. The reaction of the appropriate acetoacetates with ammonia in methanol gave the 3-aminocrotonates **6** in good yields.

Pharmacology

The compounds listed in Table I were examined for antihypertensive activities. Blood pressure was measured in unanesthetized rats with normal blood pressure (normotensive rats).[9] Some of the compounds were examined for antihypertensive activity in spontaneously hypertensive rats (SHR)[9] and the dose in mg/kg which produced a 30% drop in blood pressure was calculated from the regression line as the ED_{30} value (Table II).

Results and Discussion

The antihypertensive activities of the new dihydropyridine derivatives (I) are shown in Table I. In general, the 1,4-dihydropyridines (I), in which one carboxylate group of the 1,4-dihydropyridine-3,5-dicarboxylates was replaced by a phosphonate moiety, showed weaker antihypertensive activity than the corresponding carboxylate compounds. Among the phosphonate substituents, the diallyl phosphonate was most effective, and the activities are assumed to decrease in the following order: (R^1); allyl, crotyl, cyclopropylmethyl, iso-Pr, propargyl, 2-methoxyethyl, methyl, phenyl. Highly potent and long-lasting effects were observed with compounds bearing diallyl phosphonate in combination with the 2-(*N*-benzyl-

TABLE I. Physical and Biological Properties of 1,4-Dihydropyridine-5-phosphonates (I)

Compd. No.	R^1	R^2	R^3	Method[a]	Yield (%)	mp (°C)	Crystn.[b] solvent	Formula[c]	Antihyper-tensive[d] potency
Ar = R^3–C_6H_4									
7	Me	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	A	15	101—103	a	$C_{26}H_{32}N_3O_7P$	2
8	iso-Pr	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	A	25	113—115	b	$C_{30}H_{40}N_3O_7P$	3—4
9	C_6H_5	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	A	18	130—135	a	$C_{36}H_{36}N_3O_7P$	1
10	CH_2CH_2OMe	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	A	21	55—58	c	$C_{30}H_{40}N_3O_9P$	3
11	$CH_2CH=CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	B	17	80—82	a	$C_{30}H_{36}N_3O_7P$	4
12	$CH_2CH=CHCH_3$	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	B	2	102—104	d	$C_{32}H_{40}N_3O_7P$	3—4
13	$CH_2C\equiv CH$	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	A	36	102—103	a	$C_{30}H_{32}N_3O_7P$	3
14	CH_2–◁	$CH_2CH_2N(Me)CH_2C_6H_5$	3-NO_2	B	31	101—103[e]	c	$C_{32}H_{40}N_3O_7P$[e]	3—4
15	Me	Me	3-NO_2	B	35	221—223	d	$C_{17}H_{21}N_2O_7P$	1
16	iso-Pr	Me	3-NO_2	A	34	205—207	d	$C_{21}H_{29}N_2O_7P$	1
17	$CH_2CH=CH_2$	Me	3-NO_2	A	28	164—166	d	$C_{21}H_{25}N_2O_7P$	3
18	$CH_2CH=CH_2$	iso-Bu	3-NO_2	A	38	114—115	c	$C_{24}H_{31}N_2O_7P$	1
19	$CH_2CH=CH_2$	$CH_2CH_2NMe_2$	3-NO_2	A	12	94—96	a	$C_{24}H_{32}N_3O_7P$	2
20	$CH_2CH=CH_2$	CH_2CH_2OPr	3-NO_2	A	32	91—92	c	$C_{25}H_{33}N_2O_8P$	2
21	$CH_2CH=CH_2$	$CH_2CH_2OCH_2C_6H_5$	3-NO_2	A	37	Oil	—	$C_{29}H_{33}N_2O_8P\cdot 1/2\,H_2O$	4
22	$CH_2CH=CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	2-NO_2	A	27	Oil	—	$C_{30}H_{36}N_3O_7P$	3
23	$CH_2CH=CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	3-CF_3	B	24	Oil	—	$C_{31}H_{36}F_3N_2O_5P\cdot 1/2\,H_2O$	3
24	$CH_2CH=CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	2-CF_3	B	7	Oil	—	$C_{31}H_{36}F_3N_2O_5P$	4
25	$CH_2CH=CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	2,3-Cl_2	B	20	Oil	—	$C_{30}H_{35}Cl_2N_2O_5P\cdot 1/2\,H_2O$	4

26	$CH_2CH{=}CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	2-$OCHF_2$	A	35	Oil	—	$C_{31}H_{37}F_2N_2O_5P$	3
27	Me	Me	2-NO_2	A	39	210—211	e	$C_{17}H_{21}N_2O_7P$	4
28	$CH_2CH{=}CH_2$	Me	2-NO_2	A	31	132—133	c	$C_{21}H_{25}N_2O_7P$	4
29	$CH_2CH{=}CH_2$	Me	3-CF_3	B	28	122—124	b	$C_{22}H_{25}F_3NO_5P$	1
30	$CH_2CH{=}CH_2$	Me	2-CF_3	B	13	105—107	f	$C_{22}H_{25}F_3NO_5P \cdot 1/4\,H_2O$	4
31	$CH_2CH{=}CH_2$	Me	2,3-Cl_2	B	15	181—182	b	$C_{21}H_{24}Cl_2NO_5P$	1
32	$CH_2CH{=}CH_2$	Me	2-$OCHF_2$	A	47	129—130	f	$C_{22}H_{26}F_2NO_6P \cdot 1/2\,H_2O$	2
Ar = heterocycles									
33	$CH_2CH{=}CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$		A	46	Oil	—	$C_{28}H_{35}N_2O_6P$	2
34	$CH_2CH{=}CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$		A	27	142 (dec.)	g	$C_{29}H_{36}N_3O_5P \cdot 2HCl \cdot 5H_2O$	2
35	$CH_2CH{=}CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$	MeS	A	47	104—105	f	$C_{30}H_{38}N_3O_5PS$	3
36	$CH_2CH{=}CH_2$	$CH_2CH_2N(Me)CH_2C_6H_5$		A	41	103—104	c	$C_{30}H_{35}N_4O_6P$	3
37	$CH_2CH{=}CH_2$	Me		A	53	115—117	b	$C_{19}H_{24}NO_6P$	1

a) See the experimental section. *b*) Solvents for recrystallization: a, AcOEt–ether; b, AcOEt–hexane; c, ether; d, AcOEt; e, AcOEt–$CHCl_3$; f, ether–hexane; g, Me_2CO. *c*) All compounds were analyzed for C, H and N; the analytical results were within ±0.4% of the calculated values. *d*) Numbers have the following meanings: 1, little or no effect at 30 mg/kg; 2, effective at 30 mg/kg; 3, effective at 10 mg/kg; 4, effective at 3 mg/kg (nicardipine is effective at 1 mg/kg). The effective dose causes 25% lowering of blood pressure. *e*) Picrate ($C_6H_3N_3O_7$), for analytical sample.

TABLE II. Antihypertensive Activity of the Most Active Compounds, **11**, **24** and **25**, in SHR Compared with That of Nifedipine and Nicardipine

Compound	ED_{30} (mg/kg)[a]	Relative potency[b]
11	4.60	2.56
24	6.58	3.66
25	5.21	2.89
Nifedipine	1.50	
Nicardipine	1.80	1.00

a) See pharmacological methods. *b*) Potency relative to that of nicardipine.

N-methylamino)ethyl ester group at the 3-position and a 3-nitrophenyl, 2-trifluoromethylphenyl or 2,3-dichlorophenyl substituent at the 4-position.

Among the carboxylate substituents, the basic carboxylates mentioned above were most effective, and the 2-benzyloxyethyl ester also showed good activity. However, the methyl ester showed marked activity only when the dimethyl (**27**) or the allyl (**28**) phosphonate group was combined with a 2-nitrophenyl substituent.

Among the variations in the aryl groups at the 4-position, the compounds possessing electron-attracting groups at the 2- or 3-position showed greater activity than the corresponding heterocyclic compounds.

Some of the effective 1,4-dihydropyridines (I) were selected from those listed in Table I, and their antihypertensive activity was examined in unanesthetized SHR. The results are shown in Table II. In this series, the diallyl phosphonate compound (**11**) had the highest potency. However, it was about 2.5 times less active than nicardipine.

Structural requirements for good antihypertensive activity in this series proved to be essentially the same as those in other known 1,4-dihydropyridines except for the phosphonate moiety at the 5-position. These results indicate that the phosphonate group is comparable to the carboxylate group in terms of its effect in these compounds. Further investigations of phosphonate derivatives are in progress, and the results will be published elsewhere.

Experimental

Melting points were determined with a Büchi melting point apparatus and are uncorrected. Infrared (IR) spectra were measured on a Hitachi IR-215 spectrophotometer. NMR spectra were recorded on a Varian YX-200 spectrometer. Chemical shifts are given in δ (ppm) with tetramethylsilane as the internal standard.

Preparation of Trialkyl Phosphites (1)——The phosphites **1** were prepared in the same manner as described in the literature.[10] [R^1, bp °C (mmHg), yield %]: cyclopropylmethyl, 116—128 (3), 73; 2-methoxyethyl, 118—124 (0.5), 81.

General Procedure for Acetonylphosphonates (2)[11]——Bromoacetone (0.2 mol) was added to a preheated (70—80 °C) trialkyl phosphite **1** with stirring, and the reaction temperature was then raised to 130—150 °C. The mixture was stirred for 1—2 h at 120 °C. After cooling, the mixture was extracted twice with cold aqueous NaOH. The NaOH layer was neutralized with AcOH, and extracted twice with $CHCl_3$. The extracts were combined and washed with saturated aqueous NaCl, and dried ($MgSO_4$), then the solvent was removed *in vacuo*. The oil obtained was distilled *in vacuo* to give **2** as a colorless oil. All the acetonylphosphonates **2** thus obtained were characterized by measuring their IR and NMR spectra: IR (film): 1720 (C=O), 1260—1280 (P=O) cm^{-1}. NMR ($CDCl_3$) δ: 2.12—2.37 (3H, s, $COCH_3$), 2.97—3.34 (2H, d, J=22—23 Hz, P-CH_2). [R^1, bp °C (mmHg), yield %]: iso-Pr, 93—95 (0.5), 69; 2-methoxyethyl, 140—141 (0.8), 61; allyl, 114—116 (0.5), 51; crotyl, 128—129 (1), 41; cyclopropylmethyl, 139—141 (0.8), 42. The diphenyl ester was prepared from diphenyl methyl phosphite with bromoacetone.[11c]

Dipropargyl Acetonylphosphonate——Phosphorus trichloride (27.5 g) was added dropwise to a stirred solution of propargyl alcohol (33.6 g) and triethylamine (60.7 g) in ether (500 ml) at −10 to −5 °C. The mixture was then kept at room tempeature for 4 h. The precipitated solid was filtered off and washed with ether. The combined filtrate was refluxed for 2 h with stirring. The solvent was removed at a temperature below 10 °C, and the residue was distilled *in vacuo* to afford **3** as an oil (20.8 g, 53%), bp 138—148 °C (2 mmHg).

Warning: The unstable allene compound **3** should be distilled carefully to avoid violent reaction. IR (film): 2140, 1970, 1940 cm^{-1}. NMR ($CDCl_3$) δ: 2.50—2.80 (2H, m), 4.52—5.50 (7H, m). Next, 28% NH_4OH (62 ml) was added dropwise to a solution of the allene **3** (20.5 g) in tetrahydrofuran (THF) (100 ml) at 0—5 °C. The mixture was stirred at room temperature for 2 h. The residue obtained after evaporation of the solvent and any volatile materials *in vacuo* were purified by chromatography on silica gel with hexane–AcOEt (9 : 1, v/v) to give the dipropargyl ester of **2** (16.0 g, 71%) as a pale yellow oil, bp 131—141 °C (0.5 mmHg). IR (film): 2140, 1720 cm^{-1}. NMR ($CDCl_3$) δ: 2.37 (3H, s), 2.55—2.80 (2H, m), 3.19 (2H, d, J=23 Hz), 4.55—4.90 (4H, m).

General Procedure for 1-Arylideneacetonylphosphonates (5)——A solution of **2** (0.1 mol) and **4** (0.1 mol) in benzene (200 ml) containing a catalytic amount of piperidine–AcOH salt was refluxed for 2—24 h with continuous removal of water by the use of a Dean–Stark apparatus. The benzene solution was washed with water, aqueous NaOH, aqueous $NaHSO_3$ and water, then dried ($MgSO_4$) and concentrated to give crude **5** (containing as impurities *ca.* 20—40% benzalacetones). The residue was purified by chromatography on silica gel with hexane–AcOEt (4 : 1—1 : 4, v/v) to give **5** in 15—70% yields as a mixture of (E) and (Z)-isomers. All these **5** were characterized by measuring their IR and NMR spectra.

General Procedure for 1,4-Dihydropyridines (I)——Method A: A solution of **5** (0.01 mol) and **6** (0.01 mol) in 2-propanol (20 ml) was refluxed for 3—24 h with stirring. The solvent was removed, and the residue was purified by crystallization from solvents (AcOEt or ether) or by chromatography on silica gel with hexane–AcOEt to give the 1,4-dihydropyridines (I) in 12—47% yields. When R^2 of carboxylates was the basic ester, the residue obtained was extracted with aqueous HCl, the acid extract was extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with aqueous K_2CO_3 and water, then dried ($MgSO_4$) and concentrated to give crude (I). The residue obtained was crystallized from solvents or purified by chromatography on silica gel with hexane–AcOEt.

Method B: Purification of the crude **5** was omitted, and condensation with **6** was carried out in the same manner as in method A (2—35%).

Method C: A solution of 0.01 mol each of **2**, **4** and **6** in 2-propanol (20 ml) containing a catalytic amount of piperidine–AcOH salt was refluxed for 24 h with stirring. The solvent was removed, and the residue was treated as in method A to afford the 1,4-dihydropyridines (I) (for example, **16**) in 16% yield.

The 1,4-dihydropyridines (**7**—**37**) were prepared by method A or B. Typical examples are given below.

2-(N-Benzyl-N-methylamino)ethyl 5-Diisopropoxyphosphinyl-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine 3-Carboxylate (8)——A solution of diisopropyl 1-(3-nitrobenzylidene)acetonylphosphonate (5.33 g) and 2-(N-benzyl-N-methylamino)ethyl 3-aminocrotonate (3.73 g) in 2-propanol (75 ml) was refluxed for 3 h with stirring. The solvent was removed, then the residue was diluted with AcOEt–ether (50 ml, 1 : 1, v/v) and extracted with 50 ml of 1 N HCl then twice with 20 ml each of water. The aqueous phase was extracted twice with $CHCl_3$, and the $CHCl_3$ layer was washed with aqueous K_2CO_3 and water, then dried ($MgSO_4$) and concentrated to dryness. The residue was crystallized from AcOEt–ether to give **8** (2.64 g). Recrystallization from hexane–AcOEt gave pale yellow crystals (2.21 g, 25%), mp 113—115 °C. *Anal.* Calcd for $C_{30}H_{40}N_3O_7P$: C, 61.53; H, 6.88; N, 7.18; P, 5.29. Found: C, 61.32; H, 7.06; N, 7.14; P, 5.09. IR (KBr): 3275, 3210, 1700, 1530, 1355, 1235 cm^{-1}. NMR ($CDCl_3$) δ: 0.96 (3H, d, J=6 Hz), 1.11 (3H, d, J=6 Hz), 1.15 (3H, d, J=6 Hz), 1.25 (3H, d, J=6 Hz), 2.19 (3H, s), 2.30 (3H, d, J=2.5 Hz), 2.34 (3H, s), 2.60—2.67 (2H, m), 3.49 (2H, s), 4.08—4.59 (4H, m), 4.91 (1H, d, J=10 Hz), 5.79 (1H, d, J=5 Hz), 7.16—7.35 (6H, m), 7.64—7.72 (1H, m), 7.92—7.99 (1H, m), 8.11 (1H, t, J=2 Hz).

2-(N-Benzyl-N-methylamino)ethyl 5-Diallyloxyphosphinyl-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (11)——A solution of diallyl acetonylphosphonate (3.0 g) and 3-nitrobenzaldehyde (2.08 g) in benzene (30 ml) containing a catalytic amount of piperidine–AcOH salt was refluxed for 2 h with continuous removal of water by the use of a Dean–Stark apparatus. The benzene solution was washed with aqueous NaOH, aqueous $NaHSO_3$, water, dried ($MgSO_4$) and concentrated to give crude **5** as an oil. A solution of the oil and 2-(N-benzyl-N-methylamino)ethyl 3-aminocrotonate (3.41 g) in 2-propanol (30 ml) was refluxed for 8 h with stirring. Concentration followed by treatment as in method B gave **11** (1.38 g, 17%), as pale yellow crystals (from AcOEt–ether), mp 80—82 °C. *Anal.* Calcd for $C_{30}H_{36}N_3O_7P$: C, 61.95; H, 6.24; N, 7.22. Found: C, 61.85; H, 6.42; N, 7.27. IR (KBr): 3290, 3230, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 2.20 (3H, s), 2.31 (3H, d, J=2.5 Hz), 2.34 (3H, s), 2.64 (2H, t, J=6 Hz), 3.49 (2H, s), 3.96—4.39 [4H, m, 4.18 (2H, t-like)], 4.88 (1H, d, J=10 Hz), 4.98—5.29 (4H, m), 5.53—5.94 (2H, m), 6.17 (1H, d, J=6 Hz), 7.20—7.35 (6H, m), 7.63—7.72 (1H, m), 7.92—8.00 (4H, m), 8.10 (1H, t, J=1.5 Hz).

2-(N-Benzyl-N-methylamino)ethyl 5-Diallylphosphinyl-2,6-dimethyl-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine-3-carboxylate (24)——A solution of diallyl 1-(2-trifluoromethylbenzylidene)acetonylphosphonate (11.3 g, 21.2 mmol, *ca.* 70% purity) and 2-(N-benzyl-N-methylamino)ethyl 3-aminocrotonate (5.30 g, 21.2 mmol) in 2-propanol (120 ml) was refluxed for 48 h with stirring. The solvent was removed, and the residue was diluted with AcOEt (80 ml) and extracted with 100 ml of 1 N HCl then twice with 40 ml each of water. The aqueous phase was extracted twice with $CHCl_3$, and the $CHCl_3$ layer was washed with aqueous K_2CO_3 and water, then dried ($MgSO_4$) and concentrated to dryness. The residue was purified by chromatography on silica gel with hexane–AcOEt to give **24** (1.70 g, 13%) as a plae yellow oil. *Anal.* Calcd for $C_{31}H_{36}F_3N_2O_5P$: C, 61.58; H, 6.00; N, 4.63. Found: C, 61.50; H, 6.17; N, 4.62. IR (film): 3300, 3230, 3110, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 2.19 (3H, s), 2.17 (3H, s), 2.39 (3H, d, J=2.5 Hz), 2.60—2.67 (2H, m), 3.49 (2H, s), 3.50—3.67 (1H, m), 3.99—4.47 (5H, m), 4.82—4.96 (2H, m), 5.14

(1H, d, J=10 Hz), 5.22—5.52 (3H, m), 5.72 (1H, d, J=5 Hz), 5.80—6.01 (1H, m), 7.18—7.60 [4H, m, 7.26 (5H, s)].

Methyl 5-Diallyloxyphosphinyl-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3-carboxylate (28)——A solution of diallyl 1-(2-nitrobenzylidene)acetonylphosphonate (2.0 g) and methyl 3-aminocrotonate (0.46 g) in 2-propanol (15 ml) was refluxed for 15 h with stirring. The solvent was removed, and the residue was purified by chromatography on silica gel with hexane–AcOEt (1 : 1, v/v) to yield **28** (0.90 g, 31%) as yellow crystals (from hexane–AcOEt), mp 132—133 °C. *Anal.* Calcd for $C_{21}H_{25}N_2O_7P$: C, 56.25; H, 5.62; N, 6.25. Found: C, 56.25; H, 5.69; N, 6.22. IR (KBr): 3290, 3225, 3100, 1700 cm^{-1}. NMR ($CDCl_3$) δ: 2.29 (3H, s), 2.41 (3H, d, J=2 Hz), 3.52 (3H, s), 3.77—3.93 (1H, m), 4.13—4.31 (1H, m), 4.77—4.93 (2H, m), 5.20 (1H, d, J=10 Hz), 5.31—5.47 (2H, m), 5.53 (1H, d, J=5 Hz), 5.89—6.10 (2H, m), 7.18—7.29 (1H, m), 7.39—7.50 (1H, m), 7.55 (1H, dd, J=1.5, 8 Hz), 7.65 (1H, dd, J=1.5, 8 Hz).

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Sesquiterpene Glycosides from *Lactuca sativa* L.

NAOMI ISHIHARA, TOSHIO MIYASE,* and AKIRA UENO

Shizuoka College of Pharmacy, 2-2-1, Oshika, Shizuoka 422, Japan

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A new guaiane-type sesquiterpene glycoside, lactuside C, has been isolated from *Lactuca sativa* L. (Compositae), together with two known glycosides, lactuside A and macrocliniside A. The structure of the new compound was determined on the basis of chemical and spectral data.

Keywords——*Lactuca sativa*; Compositae; sesquiterpene glycoside; lactuside A; lactuside C; macrocliniside A

In connection with a study on the sesquiterpene glycosides of some plants in Compositae, we have also investigated *Lactuca sativa* L. (sunny lettuce). From the water extract of the roots a new guaiane-type sesquiterpene glycoside, lactuside C (2), was isolated together with two known glycosides, lactuside A (1)[1] and macrocliniside A (3).[2] The structure of the new compound was determined on the basis of chemical transformations and spectroscopic studies.

1

2: R = O-Glc

2a: R = O-Glc(OAc)$_4$

2b: R = OH

2c: R = H

3

4

5

Chart 1

TABLE I. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	1[a]	2[a]	2a[b]	2b[c]
1	6.28 (1H, br t, J=7 Hz)			
3		6.22 (1H, br s)	6.17 (1H, br s)	6.18 (1H, t, J=2 Hz)
5			3.72 (1H, br d, J=7 Hz)	3.86 (1H, br d, J=10 Hz)
6		3.58 (1H, t, J=10 Hz)	3.60 (1H, t, J=10 Hz)	3.62 (1H, t, J=10 Hz)
9			4.10 (1H, m)	4.40 (1H, dd, J=5; 2 Hz)
13a 13b	1.17 (3H, d, J=7 Hz)	1.28 (3H, d, J=7 Hz)	1.22 (3H, d, J=7 Hz)	1.25 (3H, d, J=7 Hz)
14	9.50 (1H, br s)	2.68 (3H, br s)	2.43 (3H, br s)	2.45 (3H, d, J=1 Hz)
15a 15b	2.15 (3H, br s)	2.05 (3H, br s)	2.31 (3H, br s)	2.30 (3H, br s)
Anomeric		4.87 (1H, d, J=7 Hz)	4.55 (1H, d, J=8 Hz)	
OAc			2.01, 2.02, 2.04, 2.07 (each 3H, s)	

Proton No.	2b[d]	2c[c,4]	3[a]
1			
3	6.02 (1H, t, J=1 Hz)	6.16 (1H, t, J=2 Hz)	
5	3.38 (1H, br d, J=10 Hz)	3.40 (1H, br d, J=10 Hz)	
6	2.71 (1H, t, J=10 Hz)	3.65 (1H, t, J=10 Hz)	
9	3.57 (1H, br d, J=6 Hz)		
13a 13b	0.93 (3H, d, J=7 Hz)	1.25 (3H, d, J=7 Hz)	5.45 (1H, d, J=3.1 Hz) 6.27 (1H, d, J=3.6 Hz)
14	2.31 (3H, d, J=1 Hz)	2.42 (3H, br s)	5.18 (2H, s)
15a 15b	1.95 (3H, t, J=1 Hz)	2.30 (3H, br s)	5.96 (1H, br s) 5.57 (1H, br s)
Anomeric			5.07 (1H, d, J=8 Hz)
OAc			

a) Run at 89.55 MHz in pyridine-d_5 solution. *b*) Run at 399.65 MHz in $CDCl_3$ solution. *c*) Run at 89.55 MHz in $CDCl_3$ solution. *d*) Run at 89.55 MHz in C_6D_6 solution.

Lactuside A (**1**): From the proton nuclear magnetic resonance (^{1}H-NMR) and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra, **1** was assumed to be lactuside A, which had been previously isolated from *Lactuca laciniata* MAKINO.[1] Its identity was confirmed by direct comparison of the ^{1}H-NMR, ^{13}C-NMR, infrared (IR) spectra with those of an authentic sample.[1]

Lactuside C (**2**): $C_{21}H_{28}O_9$, $[\alpha]_D^{25}+52.4°$ was obtained as an amorphous powder. Its ultraviolet (UV) spectrum showed an absorption maximum at 256 (4.12) nm (α,β-unsaturated carbonyl). The IR spectrum showed hydroxyl (3450 cm^{-1}), γ-lactone ring (1780 cm^{-1}), α,β-α',β'-unsaturated ketone (1685 cm^{-1}) and double bond (1620 cm^{-1}) absorptions. The ^{1}H-NMR spectrum showed a doublet methyl signal at δ 1.28 (3H, d, J=7 Hz), two vinyl methyl signals at δ 2.05 (3H, br s) and 2.68 (br s) and an olefinic proton signal at δ 6.22 (1H, br s), suggesting that **2** has a lactucin-type skeleton.[1] In the ^{13}C-NMR spectrum, twenty-one signals were observed, including the signals of a glucopyranosyl residue, and acid hydrolysis of **2** afforded glucose as the sugar moiety. Acetylation of **2** afforded a tetraacetate (**2a**). The ^{13}C-NMR spectrum of **2** was similar to that of crepidiaside B, which had been isolated from *Crepidiastrum keiskeanum* NAKAI,[3] but the C-7 signal (δ 46.5) of **2** was shifted upfield by 9.1 ppm, while the C-8 (δ 29.5) and C-9 (δ 80.6) signals were shifted downfield by 3.6 and

TABLE II. ^{13}C-NMR Chemical Shifts

Carbon No.	1	2	(Δ_{2-4})	(Δ_{2-5})	3	4[3]	5[3]
Aglycone moiety							
1	150.2	132.9			41.6	131.7	136.5
2	33.0	196.7			37.5	195.0	37.5
3	79.9	135.4			80.9	134.4	129.0
4	137.0	171.6			151.0	169.5	142.2
5	127.1	51.3			49.5	49.8	52.3
6	80.3	84.3		(+1.8)	84.6	83.8	82.5
7	49.5	46.5	(−9.1)	(−16.4)	36.8	55.6	62.9
8	22.5	29.5	(+3.6)	(−40.9)	40.0	25.9	70.4
9	25.5	80.6	(+43.1)	(+34.1)	72.5	37.5	46.5
10	145.7	150.1	(−2.7)		153.4	152.8	126.9
11	41.4	40.9			141.2	41.3	42.2
12	178.6	177.9			170.3	177.2	178.5
13	12.9	12.4			119.2	12.2	16.1
14	195.8	19.8[a]			111.0	21.4	23.0
15	11.3	19.5[a]			112.3	68.8	68.2
Sugar moiety							
1′	102.2	103.5			104.7	104.1	103.2
2′	75.1	75.1			75.5	75.0	75.1
3′	78.6	78.6			78.7	78.3	78.6
4′	71.8	71.6			72.0	71.5	71.8
5′	78.6	78.6			78.5	78.1	78.2
6′	62.9	62.7			63.1	62.6	62.5

Run at 22.5 MHz in pyridine-d_5 solution. *a*) Assignments may be interchanged.

43.1 ppm, respectively. From these data, it was assumed that **2** had a glucose moiety at C-9 and three methyl groups at C-13, C-14 and C-15. Enzymatic hydrolysis of **2** afforded **2b** as an aglycone. In the ^{1}H-NMR spectrum of **2b** in $CDCl_3$, the H-9 and H-5 signals were observed at δ 4.40 (1H, dd, J=5, 2 Hz) and 3.86 (1H, br d, J=10 Hz), respectively. The H-5 signal showed a downfield shift by 0.46 ppm compared with that of desacetoxymatricarin (**2c**).[4] This shift requires that the hydroxyl group has the same spatial direction as H-5. If it is assumed that all naturally occurring sesquiterpene lactones from Compositae plants have α-oriented H-7 on the basis of biosynthetic considerations, the C-9 hydroxyl group and H-5 are α-oriented in **2b**. The C-13 methyl signal showed an upfield shift by 0.32 ppm in benzene-d_6 relative to chloroform-d solution. Therefore, **2b** has a pseudo-equatorial (α) methyl group at C-11 on the basis of Narayanan's rule.[5] Thus, the structure of lactuside C was decided to be **2**.

Macrocliniside A (3): By comparing the ^{1}H- and ^{13}C-NMR spectra, **3** was shown to be identical with macrocliniside A, which had been isolated from *Macroclinidium trilobum* MAKINO.[2]

Experimental

Optical rotations were determined with a JASCO DIP-140 digital polarimeter. IR spectra were taken on a JASCO A-202 infrared spectrophotometer and UV spectra on a shimadzu UV-360 recording spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL FX 90Q (89.55 and 22.5 MHz, respectively) and JEOL GX-400 (399.65 MHz) spectrometers. Chemical shifts are given on the δ (ppm) scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Gas chromatography (GC) was run on a Hitachi K 53 gas chromatograph. High-performance liquid chromatography (HPLC) was run on a Kyowa Seimitsu model K880 instrument.

Isolation——Fresh roots (8.5 kg) of *Lactuca sativa* L. collected in spring 1986, in Shizuoka, Japan, were

extracted twice with hot water. The crude extract was passed through an Amberlite XAD-2 column, and the eluate with MeOH was concentrated under reduced pressure. The residue (30 g) was chromatographed repeatedly on a silica gel column with a chloroform–methanol system and subjected to HPLC (Develosil ODS-10) with a water–acetonitrile system, yielding three sesquiterpene glycosides.

Lactuside A (1)——Amorphous powder (148 mg) (0.0017%). IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1685. ^{1}H- and ^{13}C-NMR; Tables I and II.

Lactuside C (2)——Amorphous powder (32 mg) (0.0004%), $[\alpha]_D^{25}+52.4°$ (c=0.82, MeOH). *Anal.* Calcd for $C_{21}H_{28}O_9$: C, 59.42; H, 6.65. Found: C, 59.61; H, 6.52. IR ν_{max}^{KBr} cm^{-1}: 3450, 1780, 1685, 1620. UV λ_{max}^{MeOH} nm (log ε): 256 (4.12). ^{1}H- and ^{13}C-NMR: Tables I and II.

Macrocliniside A (3)——Amorphous powder (9 mg) (0.0001%), $[\alpha]_D^{23}-4.6°$ (c=0.87, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3450, 1760. ^{1}H- and ^{13}C-NMR: Tables I and II.

Acetylation of Lactuside C (2)——Compound **2** (8 mg) was dissolved in pyridine and acetic anhydride (each 0.1 ml) and the mixture was left at room temperature overnight. The reagents were evaporated off *in vacuo* to give the tetraacetate (**2a**) (6 mg) as an amorphous powder, after purification by HPLC [Develosil ODS-10, H_2O–CH_3CN (55:45)] (6 mg). ^{1}H-NMR: Table I.

Enzymatic Hydrolysis of Lactuside C (2)——Compound **2** (2 mg) was dissolved in water (0.1 ml) and treated with cellulase for 18 h at 37 °C with stirring. This solution was extracted with ether and the ether layer was concentrated *in vacuo* to give the aglycone (**2b**) (0.2 mg) as an amorphous powder. MS m/z: 262 (M^+, 100), 244 (43), 150 (80). ^{1}H-NMR: Table I.

Acid Hydrolysis of Lactuside C (2)——A solution of the glycoside (*ca.* 1 mg) in 10% H_2SO_4 (2 drops) was heated in a boiling water bath for 30 min. The solution was passed through an Amberlite IR-45 column and concentrated to give a residue, which was reduced with $NaBH_4$ (*ca.* 2 mg) for 1 h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and concentrated to dryness. Boric acid was removed by distillation with methanol. The residue was acetylated with acetic anhydride and pyridine (1 drop each) at 100 °C for 1 h and the reagents were evaporated off *in vacuo*. Glucitol acetate was detected by GC. GC conditions: column, 1.5% OV-17 (3 mm × 1 m); column temperature, 200 °C; carrier gas, N_2; t_R, 8.1 min.

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Flavonoids Syntheses. VI.[1] Synthesis and Spectral Properties of 4-Arylcoumarins (Neoflavones)

MUNEKAZU IINUMA,*,[a] TOSHIYUKI TANAKA,[a] KOJI HAMADA,[a] MIZUO MIZUNO[a] and FUJIO ASAI[b]

Gifu Pharmaceutical University,[a] 6–1 Mitahora-higashi 5-chome, Gifu 502, Japan and Department of Liberal Science, Aichi Gakuin University,[b] Iwasaki, Nisshin-cho, Aichi 470–01, Japan

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Eight neoflavones were synthesized for examination of their spectral properties. In the mass spectra, 2′-oxygenated neoflavones showed characteristic fragments owing to dehydroxylation or demethoxylation. Other spectral data ultraviolet, proton and carbon-13 nuclear magnetic resonance, however, showed no specific features that could be used to characterize the structures.

Keywords——neoflavone synthesis; 4-arylcoumarin; 2′-oxygenated neoflavone; mass spectrum

4-Arylcoumarins can be regarded as derivatives of flavonoids (C_6–C_3–C_6) on the basis of their biosynthetic pathway[2] and their positive color reaction in the Shinoda test (Mg + HCl)[3]; they are sometimes called neoflavones. These compounds are found in nature in Leguminosae (*Dalbergia*[4] and *Macherium* spp.[5]) and in Rubiaceae (*Exostemma*[6] and *Coutarea* spp.[7]). To date, the spectroscopic properties of neoflavones have not been investigated in detail. In this paper, we present some spectral data on neoflavones with simple substitution patterns (**1**—**8**) (Chart 1) in order to clarify the differences from those of the relevant flavones.

1: $R_1 = R_2 = R_3 = R_4 = H$
2: $R_1 = OH$, $R_2 = R_3 = R_4 = H$
3: $R_2 = OH$, $R_1 = R_3 = R_4 = H$
4: $R_3 = OH$, $R_1 = R_2 = R_4 = H$
5: $R_1 = R_4 = OH$, $R_2 = R_3 = H$
6: $R_1 = OMe$, $R_2 = R_3 = R_4 = H$

7: $R_5 = H$
8: $R_5 = OH$

Chart 1

Synthesis of Eight Neoflavones

The benzoylacetic acid ethyl esters required for preparation of the above neoflavones, *i.e.*, the unsubstituted compound (**9**), 2-isopropyloxy- (**10**), 3-hydroxy- (**11**), 4-hydroxy- (**12**), 2,5-diisopropyloxy- (**13**), 2-methoxy- (**14**), and 3-isopropyloxybenzoylacetic acid ethyl ester (**15**), were synthesized from the corresponding acetophenones by condensation with ethyl carbonate in the presence of sodium hydride. The resultant esters were condensed with

TABLE I. Mass Spectral Data for 1—8

	m/z (rel. int.)
1	254 [M$^+$] (100), 226 (99), 225 (12.5), 197 (4.3)
2	270 [M$^+$] (47.6), 253 (14.2), 252 (100), 225 (14.9)
3	270 [M$^+$] (100), 242 (70.4), 213 (31.7)
4	270 [M$^+$] (100), 242 (82), 213 (31.7)
5	286 [M$^+$] (7.8), 269 (47.4), 268 (100), 240 (52.9), 212 (57.9), 184 (18.4), 155 (13.2)
6	284 [M$^+$] (100), 256 (17), 253 (58.5), 242 (34.1), 235 (9.7)
7	238 [M$^+$] (94.7), 210 (100), 181 (34.2), 165 (7.8), 152 (23.7)
8	254 [M$^+$] (79.1), 226 (100), 198 (23.2), 181 (11.6)

TABLE II. ^{13}C-NMR Spectral Data for Synthetic Neoflavones (1—5, 7 and 8)

Carbon number	1	2	3	4	5	7	8
2	159.2	160.5	160.1	160.2	160.3	160.1	160.2
3	110.2	110.1	109.9	114.2	109.9	110.3	110.3
4	156.8	154.4	156.9	156.9	157.5	155.5	157.5
5	157.1	156.6	157.2	157.2	156.5	128.0	129.9
6	100.7	99.0	99.3	99.3	98.9	113.2	113.2
7	161.8	161.4	161.8	161.8	161.2	161.5	161.4
8	94.7	94.4	94.8	94.8	94.2	102.6	102.7
9	156.8	157.6	156.9	156.9	157.5	155.4	155.5
10	99.2	102.6	100.8	100.8	101.9	110.6	110.7
1′	139.7	127.9	140.9	130.1	127.9	135.2	136.5
2′	127.3	154.2	114.5$^{a)}$	129.2	154.5	128.3$^{a)}$	115.1
3′	127.4	114.2	156.5	114.2	115.1$^{a)}$	128.9$^{b)}$	155.5
4′	127.8	128.9	114.9$^{a)}$	157.6	115.3$^{a)}$	129.5	116.5
5′	127.4	118.4	128.6	114.2	154.2	128.9$^{b)}$	128.2
6′	127.3	128.3	118.2	129.2	114.7	128.3$^{a)}$	118.9

All spectra were measured in DMSO-d_6. Assignments bearing the same superscript in any column may be reversed.

phloroglucinol or resorcinol by means of the Pechamann reaction under dry hydrogen chloride to give 5,7-dihydroxy- (**1**), 5,7-dihydroxy-2′-isopropyloxy- (**16**), 3′,5,7-trihydroxy- (**3**), 4′,5,7-trihydroxy- (**4**), 5,7-dihydroxy-2′,5′-diisopropyloxy- (**17**), 5,7-dihydroxy-2′-methoxy- (**6**), 7-hydroxy-3′-isopropyloxy- (**18**) and 7-hydroxyneoflavone (**7**), respectively. Further, **16**, **17** and **18** were deisopropylated with boron trichloride to give 2′,5,7-trihydroxy- (**2**), 2′,5,5′,7-tetrahydroxy- (**5**) and 3′,7-dihydroxyneoflavone (**8**). Among these compounds, **1** and **4** have been described by other investigators.[7a,8] Tables I and II show the mass spectral (MS) data and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data of the neoflavones thus obtained.

Spectral Properties of the Neoflavones

Ultraviolet (UV) and proton nuclear magnetic resonance (^{1}H-NMR) spectral data of the neoflavones are given in Experimental. In addition to the bathochromic shifts in the UV after addition of some reagents, two absorption bands, called bands I and II, due to cinnamoyl and benzoyl moieties of the flavone skeleton, are widely utilized to determine the positions of hydroxy groups.[9] These bands, however, were not observed in the case of the neoflavones. In neoflavones there seems to be no mesomeris effect between the A and B rings. No bathochromic shifts were observed on addition of AcONa and $AlCl_3$, or MeONa. The latter

−CO

−OR (ROH)

R=H or Me

Chart 2

results in degradation of the coumarin ring.

In the ^{1}H-NMR spectra of flavones, the protons at C-2′ and C-6′ are observed at rather low field, but in the neoflavones all protons of the B ring moiety appear at similar positions, which causes some difficulty in distinguishing the substitution patterns (for example, dioxygenation at C-3′,4′ or at C-2′,5′).[10)]

In the MS of neoflavones, two noteworthy results were obtained. As shown in Chart 2, the neoflavones lacking an O-function at C-2′ such as **1**, **3**, **4**, **7** and **8**, showed stable fragments of $[M^+-28]$ caused by decarboxylation. On the other hand, in the case of 2′-oxygenated neoflavones such as **2**, **5** and **6**, dehydroxylation or demethoxylation between C_5-OH and substituents on C-2′ occurred to give $[M^+-17]$ or $[M^+-59]$. These characteristic fragmentations have led us to propose a revised structure for 2,5,5′-trihydroxy-7-methoxyneoflavone isolated from *Coutarea hexandra*.[11)]

In the ^{13}C-NMR spectra, all carbons were assigned on the basis of substituent-induced shifts due to hydroxy groups. The chemical shifts of the A and B ring moieties could be independently considered as follows: the chemical shifts of the coumarin moiety of **7**[12a)] correspond to those of 7-hydroxycoumarin[12b)] except that of C-4, which is shifted down-field (*ca.* 11 ppm) by the presence of the B ring. On the other hand, the chemical shifts of the B ring moiety can be assigned by comparison with those of simple benzene derivatives. In the case of flavones, hydroxy or methoxy groups attached at C-2′ affect the chemical shift of the C-3 carbon (*ca.* +3 ppm).[13)] In spite of the similar circumstances, a hydroxy group at C-2′ in neoflavone had little influence on the chemical shifts of C-3 and C-5 (C-3, 0 ppm; C-5, −0.6 ppm).

Experimental

Melting points were determined on a Büchi melting point apparatus and are uncorrected. UV spectra were taken on a Hitachi 323 spectrometer. MS were obtained on a JEOL JMS-300 mass spectrometer at 70 eV. ^{1}H-NMR spectra were taken on a Hitachi R-20B at 60 MHz and chemical shifts are given in δ value (ppm) with tetramethylsilane as an internal standard. ^{13}C-NMR spectra were obtained on a JEOL FX 90 spectrometer operating at 27.5 MHz, spectral width 5000 Hz and 4096 data points. BW-820 MH (Fuji Devison Chemicals, Ltd.) was used for column chromatography.

General Procedures for Synthesis of Benzoylacetic Acid Ethyl Esters——A solution of an acetophenone (0.02 mol) dissolved in dry ether (20 ml) was added dropwise to an ethereal solution containing diethyl carbonate (0.01 mol) and sodium hydride (0.05 mol 60% dispersion in mineral oil). The solution was stirred at room temperature, and then refluxed for 7 h. The reaction mixture was poured into water, acidified with hydrochloric acid (2 N), and extracted with AcOEt. The AcOEt extract was purified by column chromatography on silica gel with $CHCl_3$ to give a benzoylacetic acid ethyl ester.

General Procedures for Pechmann Condensation——An ethanol solution (30 ml) containing phloroglucinol (or resorcinol) (0.02 mol) and a benzoylacetic acid ester (0.02 mol) obtained by the procedure mentioned above was saturated with HCl gas under cooling in an ice bath. The reaction mixture was left at room temperature for 3 d, then poured into water and extracted with AcOEt. The AcOEt extract was purified by recrystallization or by column chromatography on silica gel with EtOAc–C_6H_{14} to yield a neoflavone.

5,7-Dihydroxyneoflavone (1)——A colorless powder, mp 229—230 °C (AcOEt) (lit.[7a)] mp 234—235 °C), yield 2.5 g (53%). *Anal.* Calcd for $C_{15}H_{10}O_4$: C, 70.86; H, 3.96. Found: C, 70.81; H, 4.20. ^{1}H-NMR (DMSO-d_6) δ: 5.78

(1H, s, H-3), 6.21, 6.33 (1H, each d, J=2.3 Hz, H-6 and 8), 7.40 (5H, s, C_6H_5), 10.03, 10.30 (1H, each s, OH). UV λ_{max}^{MeOH} nm (log ε): 262 (4.1), 334 (4.1). λ^{+NaOMe}: 285 sh, 382, (dec.). λ^{+AlCl_3}: 261, 335. $\lambda^{+AlCl_3/HCl}$: 261, 335. λ^{+AcONa}: 270, 384. λ^{+AcONa/H_3BO_3}: 261, 335.

2′,5,7-Trihydroxyneoflavone (2)——2-Isopropyloxyacetophenone: a yellow oil. ^{1}H-NMR (CCl_4) δ: 1.24 (6H, d, J=6 Hz, ($C\underline{H}_3)_2$CH), 2.38 (3H, s, Ac), 4.86 (1H, hep, >C$\underline{H}$). 2-Isopropyloxybenzoylacetic acid ethyl ester (**10**): a pale brown oil, yield 3.9 g (77%). ^{1}H-NMR (CCl_4) δ: 1.20 (3H, t, J=6 Hz, $CH_2C\underline{H}_3$), 1.39 (6H, d, J=6 Hz, ($C\underline{H}_3)_2$CH), 3.78 (2H, s, $COCH_2CO$), 4.02 (2H, q, J=6 Hz, $C\underline{H}_2CH_3$), 4.60 (1H, hep, J=6 Hz, >C$\underline{H}$). 5,7-Dihydroxy-2′-isopropyloxyneoflavone (**16**): a pale yellow powder, mp 189 °C (dec.) (AcOEt–C_6H_{14}), yield 4 g (67%). ^{1}H-NMR (DMSO-d_6) δ: 1.18 (6H, d, J=6 Hz, ($C\underline{H}_3)_2$CH), 5.73 (1H, s, H-3), 6.20, 6.30 (1H, each d, J=3.0 Hz, H-6 and 8), 6.83—7.60 (4H, m, B ring protons), 9.73, 10.05 (1H, s, OH). **16** was treated with BCl_3 to obtain **2** as a pale yellow powder, mp 249—250 °C (AcOEt–C_6H_{14}). *Anal.* Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.42; H, 4.01. ^{1}H-NMR (DMSO-d_6) δ: 5.63 (1H, s, H-3), 6.13, 6.25 (1H, each d, J=3 Hz, H-6 and 8), 6.63—7.28 (4H, m, B ring protons), 9.30 (1H, s, OH), 10.35 (2H, brs, 2×OH). UV λ_{max}^{MeOH} nm (log ε): 261 (3.9), 330 (4.0). λ^{+NaOMe}: 268, 384, (dec.). λ^{+AlCl_3}: 260 sh, 331. $\lambda^{+AlCl_3/HCl}$: 260 sh, 331. λ^{+AcONa}: 268, 379. λ^{+AcONa/H_3BO_3}: 262 sh, 328.

3′,5,7-Trihydroxyneoflavone (3)——A pale yellow powder, mp 260 °C (AcOEt–C_6H_{14}), yield 3.7 g (68%). *Anal.* Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.32; H, 3.89. ^{1}H-NMR (DMSO-d_6) δ: 5.68 (1H, s, H-3), 6.15, 6.25 (1H, each d, J=3 Hz, H-6 and 8), 6.68—7.43 (4H, m, B ring protons), 9.95 (1H, s, OH), 10.32 (2H, s, 2×OH). UV λ_{max}^{MeOH} nm (log ε): 259 (4.1), 334 (4.1). λ^{+NaOMe}: 268 sh, 380, (dec.). λ^{+AlCl_3}: 259, 335. $\lambda^{+AlCl_3/HCl}$: 258, 334. λ^{+AcONa}: 271 sh, 379. λ^{+AcONa/H_3BO_3}: 259, 335.

4′,5,7-Trihydroxyneoflavone (4)——Colorless rectangles, mp 260 °C (AcOEt) (lit.[6a] mp 294—295 °C (dec.)), yield 2.8 g (52%). *Anal.* Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.91; H, 4.02. ^{1}H-NMR (DMSO-d_6) δ: 5.68 (1H, s, H-3), 6.15, 6.25 (1H, each d, J=3 Hz, H-6 and 8), 6.73 (2H, d, J=8 Hz, H-3′ and 5′), 7.15 (2H, d, J=8 Hz, H-2′ and 6′), 9.48, 9.93, 10.15 (1H, each s, OH). UV λ_{max}^{MeOH} nm (log ε): 263 (4.0), 335 (4.3). λ^{+NaOMe}: 275, 373, (dec.). λ^{+AlCl_3}: 262, 333. $\lambda^{+AlCl_3/HCl}$: 261, 333. λ^{+AcONa}: 275, 315, 380. λ^{+AcONa/H_3BO_3}: 262, 333.

2′,5,5′,7-Tetrahydroxyneoflavone (5)——2,5-Diisopropyloxybenzoylacetic acid ethyl ester (**13**): a pale yellow oil, yield 4 g (65%). ^{1}H-NMR (CCl_4) δ: 1.23 (3H, t, J=6 Hz, $CH_2C\underline{H}_3$), 1.30, 1.35 (6H, each d, J=6 Hz, ($C\underline{H}_3)_2$CH), 3.86 (2H, s, $COCH_2CO$), 4.12 (2H, q, J=6 Hz, $C\underline{H}_2CH_3$), 4.40—4.52 (2H, m, 2×CH). 5,7-Dihydroxy-2′,5′-diisopropyloxyneoflavone (**17**): a pale yellow oil, yield 4.1 g (56%). ^{1}H-NMR ($CDCl_3$) δ: 1.10, 1.28 (6H, each d, J=6 Hz, ($C\underline{H}_3)_2$CH), 4.50—4.55 (2H, m, 2×CH), 5.80 (1H, s, H-3), 6.13, 6.35 (1H, each d, J=3 Hz, H-6 and 8), 6.75 (3H, brs, H-3′,4′ and 6′). **17** was treated with BCl_3 to give **5** as yellow rectangles, mp 280—285 °C (AcOEt). *Anal.* Calcd for $C_{15}H_{10}O_6$: C, 62.94; H, 3.52. Found: C, 63.06; H, 3.81. ^{1}H-NMR (DMSO-d_6) δ: 5.70 (1H, s, H-3), 6.13, 6.41 (1H, each d, J=3 Hz, H-6 and 8), 6.50—6.66 (3H, m, H-3′,4′ and 6′). UV λ_{max}^{MeOH} nm (log ε): 261 (4.1), 333 (3.9). λ^{+NaOMe}: 270 sh, 387, (dec.). λ^{+AlCl_3}: 261, 333. $\lambda^{+AlCl_3/HCl}$: 261, 333. λ^{+AcONa}: 271, 383. λ^{+AcONa/H_3BO_3}: 261, 335.

5,7-Dihydroxy-2′-methoxyneoflavone (6)——2-Methoxybenzoylacetic acid ethyl ester (**14**): a colorless oil, yield, 2.2 g (49%). ^{1}H-NMR (CCl_4) δ: 1.13 (3H, t, J=6 Hz, $CH_2C\underline{H}_3$), 3.78 (3H, s, OMe), 3.88 (2H, s, $COCH_2$), 4.04 (2H, q, J=6 Hz, $C\underline{H}_2CH_3$), 6.78—7.55 (4H, m, B ring protons). **6**: mp 178—180 °C (dec.) (AcOEt), a pale yellow powder, yield 3.2 g (56%). *Anal.* Calcd for $C_{16}H_{12}O_5$: C, 67.60; H, 4.26. Found: C, 67.68; H, 4.11. ^{1}H-NMR (DMSO-d_6) δ: 3.63 (3H, s, OCH_3), 5.63 (1H, s, H-3), 6.05, 6.20 (1H, each d, J=2.5 Hz, H-6 and 8), 6.83—7.45 (4H, m, B ring protons), 9.73, 10.08 (1H, each s, OH). UV λ_{max}^{MeOH} nm (log ε): 261 (4.1), 330 (4.0). λ^{+NaOMe}: 275, 381, (dec.). λ^{+AlCl_3}: 261, 331. $\lambda^{+AlCl_3/HCl}$: 261, 330. λ^{+AcONa}: 254, 378. λ^{+AcONa/H_3BO_3}: 261, 331.

7-Hydroxyneoflavone (7)——Colorless needles, mp 230—235 °C (EtOH), yield 2.5 g (52%). *Anal.* Calcd for $C_{15}H_{10}O_3$: C, 75.62; H, 4.23. Found: C, 75.52; H, 4.39. ^{1}H-NMR (DMSO-d_6) δ: 6.05 (1H, s, H-3), 6.80—7.38 (3H, m, H-5, 6 and 8), 7.55 (5H, brs, C_6H_5), 10.20 (1H, s, OH). UV λ_{max}^{MeOH} nm (log ε): 238 (4.1), 330 (4.2). λ^{+NaOMe}: 254, 379, (dec.). λ^{+AlCl_3}: 257, 330. $\lambda^{+AlCl_3/HCl}$: 258, 330. λ^{+AcONa}: 254, 378. λ^{+AcONa/H_3BO_3}: 257, 331.

3′,7-Dihydroxyneoflavone (8)——3-Isopropyloxybenzoylacetic acid ethyl ester (**15**): a pale brown oil, yield 4.1 g (82%). ^{1}H-NMR (CCl_4) δ: 1.20 (3H, t, J=6 Hz, $CH_2C\underline{H}_3$), 1.30 (6H, d, J=6 Hz, ($C\underline{H}_3)_2$CH), 3.85 (2H, s, $COCH_2CO$), 4.15 (2H, q, J=6 Hz, $C\underline{H}_2CH_3$), 4.60 (1H, hep, J=6 Hz, >CH). 7-Hydroxy-3′-isopropyloxyneoflavone (**20**): a colorless powder, mp 120 °C (EtOH), yield 3.7 g (63%). ^{1}H-NMR (CCl_4) δ: 1.71 (6H, d, J=6 Hz, ($C\underline{H}_3)_2$CH), 4.45 (1H, hept., $(CH_3)_2C\underline{H}$), 6.25 (1H, s, H-3), 6.83—7.15 (7H, m, aromatic protons). **8**: mp 260 °C (EtOH), a colorless powder. *Anal.* Calcd for $C_{15}H_{10}O_4$: C, 70.86; H, 3.96. Found: C, 70.80; H, 4.01. ^{1}H-NMR (DMSO-d_6) δ: 6.10 (1H, s, H-3), 6.62—7.45 (7H, m, aromatic protons), 9.70, 10.50 (1H, each s, OH). UV λ_{max}^{MeOH} nm (log ε): 238 (4.3), 330, (4.2). λ^{+NaOMe}: 244, 376, (dec.). λ^{+AlCl_3}: 238, 328. $\lambda^{+AlCl_3/HCl}$: 238, 328. λ^{+AcONa}: 242, 378. λ^{+AcONa/H_3BO_3}: 273 sh, 330.

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Determination of Aspirin and Salicylic Acid in Aspirin Suppositories by Second Derivative Ultraviolet Spectrometry

KEISUKE KITAMURA,* MASANARI MABUCHI, TOYOKO FUKUI, and KEIICHIRO HOZUMI

Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan

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Aspirin and salicylic acid in aspirin suppositories were assayed by second derivative ultraviolet spectrometry without any procedure for extraction of the pharmaceuticals from the suppository bases or for separation. The results coincided well with those obtained by the USP method. The contents of aspirin and salicylic acid could be determined with confidence limits (95%) of <0.2% for aspirin, and <0.02% for salicylic acid.

Keywords——derivative spectrometry; second derivative spectrum; aspirin; aspirin suppository; salicylic acid

Aspirin and its major decomposition product, salicylic acid, in aspirin suppositories are generally determined by the USP method.[1,2] However, the process of column chromatographic separation of aspirin and salicylic acid from suppository bases is very troublesome, and furthermore this time-consuming process may cause the hydrolysis of aspirin to salicylic acid.

High-performance liquid chromatographic (HPLC) methods[3,4] have been reported, but the ethanol extraction of aspirin from suppository bases was incomplete,[3] and warming the suppositories in methanol for the extraction would promote the hydrolysis of aspirin.[3,5,6]

We have recently reported the quantitative determination of aspirin and salicylic acid in aspirin powder[6] and in aspirin tablets[7] by second derivative ultraviolet (UV) spectrometry without any procedure for separation of the pharmaceuticals or elimination of tablet excipients. In this paper, we show that the second derivative UV spectrometry is also applicable to the assay of aspirin suppositories; the procedure is simple and rapid, and does not require any procedure of extraction or separation.

Experimental

Solvents——Solvent A: A 1% (w/v) solution of monochloroacetic acid in chloroform was used as the solvent for dissolution of suppositories. Solvent B: A 1% (w/v) solution of citric acid in ethanol was used as the solvent for dilution and measurement of spectra, since solvent A (chloroform) has absorption up to nearly 250 nm. Monochloroacetic acid and citric acid were used to suppress the hydrolysis of aspirin.[6,7]

Preparation of Assay Solutions——Method I: Five aspirin suppositories were transferred into a 25-ml volumetric flask, dissolved and diluted to volume with solvent A. Then a 1.0-ml aliquot of the solution was diluted to 25.0 ml with solvent B for the assay of salicylic acid. For the assay of aspirin, a 2.0-ml aliquot of the solution just prepared for the assay of salicylic acid was further diluted to 25.0 ml.

Method II: The sample prepared according to the USP method was diluted with solvent B. The details are as follows. Twenty aspirin suppositories were melted, stirred, and cooled, and then an aliquot of the treated suppositories, equivalent to about 250 mg of aspirin, was accurately weighed, dissolved, and diluted to a concentration of about 1.0 mg/ml with chloroform containing 2% HCl–methanol (1 : 50) solution. A 3.0-ml aliquot of the solution was diluted to 50.0 ml with solvent B to measure the second derivative spectrum for aspirin assay.

For assay of salicylic acid, an accurately weighed aliquot of the melted suppositories, equivalent to about 50 mg of aspirin, was transferred into a 10-ml volumetric flask, dissolved and diluted to volume with solvent A. Then, a 2.0-ml aliquot of the solution was diluted to 25.0 ml with solvent B.

Second Derivative UV Spectrometry——A derivative spectrophotometry unit (Shimadzu DES-2) was connected to a UV-VIS double-beam scanning spectrophotometer (Shimadzu UV-210A). Second derivative spectra were obtained in 1-cm cells at a slit width of 1 nm, a scanning speed of 480 nm/min, a response of 1.0, and the derivative wavelength difference $\Delta\lambda$ of 2 nm.

Results and Discussion

Cacao butter, Witepsol, macrogol, *etc.*, which are generally adopted as suppository bases, do not have absorptions in the range of 250—400 nm. However, in some cases, coloration of the bases (sample D) and insoluble materials contained in the bases (sample C) caused signal background in zero order spectra. They are entirely eliminated in second derivative spectra, giving a flat base line. Typical second derivative spectra of aspirin and salicylic acid in sample solutions are shown in Fig. 1.

Concentrations of aspirin and salicylic acid were determined from D_a and D_s values (Fig. 1), respectively, based on linear calibration curves obtained with the derivative unit DES-2 as described in the previous paper,[7] and since the D_a value decreased linearly with the increasing concentration of coexisting salicylic acid (C_s μg/ml), the aspirin concentration was corrected for C_s as also previously reported.[7]

Even though an assay sample was prepared by melting twenty suppositories with stirring according to the USP method, a slight nonuniformity of aspirin content was still present between different aliquots of the sample. Thus, to compare the results of the derivative method with the USP method exactly, the same aliquot was used for both methods, as described in method II. That is, the solution of an aliquot was assayed by the USP method three times using three columns at the same time, while three assay solutions were prepared

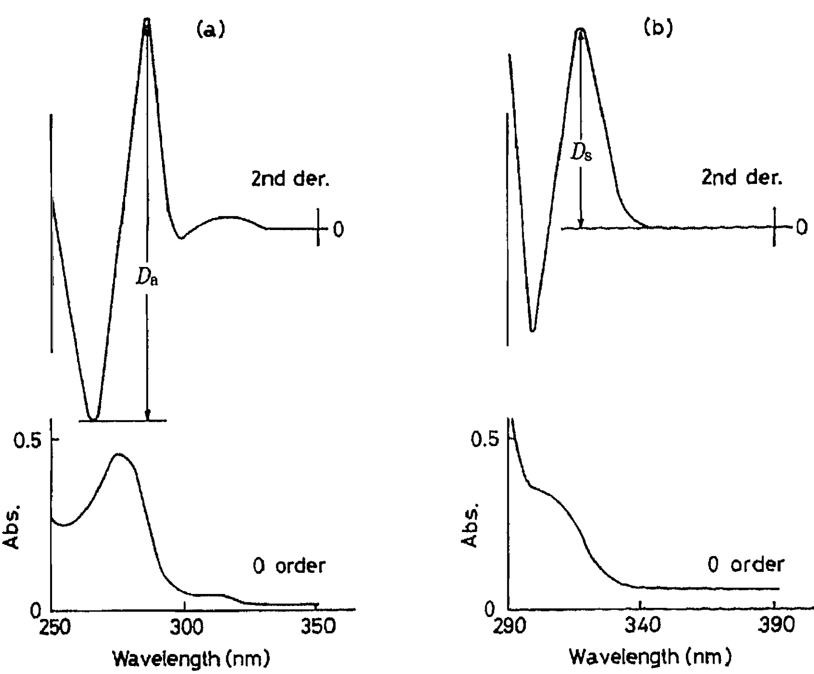

Fig. 1. Zero Order and Second Derivative UV Spectra of Aspirin and Salicylic Acid in Assay Solutions of Sample C

(a) For aspirin assay. (b) For salicylic acid assay.

TABLE I. Assay Results of Aspirin in Commercial Suppositories

Suppository	Amount of aspirin[a] (mg)	Aspirin found by deriv. method (%)[b]	Aspirin found by USP method (%)[c]
A	243.2	100.2±0.1	100.1±0.6
	243.4	99.8±0.2	99.0±0.7
	251.3	99.9±0.1	99.3±3.0
B	255.5	98.6±0.1	98.7±0.6
	247.6	98.5±0.1	98.0±1.0
	247.6	97.6±0.2	96.2±1.0
C	250.5	104.0±0.1	103.0±1.0
	252.4	105.1±0.1	104.2±0.0
	250.4	96.2±0.1	95.8±1.0
D	273.5	107.2±0.2	106.8±1.4
	245.3	106.5±0.1	105.9±2.1
	255.8	107.1±0.1	106.8±1.0

a) Amount of claimed aspirin content in a sample aliquot of the melted suppository. *b*) Mean value with confidence limits at the 95% level (n=15, 5 scans each for 3 solutions). *c*) Mean value with confidence limits at the 95% level (n=3).

TABLE II. Assay Results of Salicylic Acid in Commercial Suppositories

Suppository	Salicylic acid found by deriv. method (%)	Salicylic acid found by USP method (%)
A	3.59±0.01[a]	3.62
	3.58±0.01	3.61
	3.61±0.02	3.57
	3.66±0.02	3.81
	3.55±0.02	3.59
	m=3.60	m=3.64
B	0.95±0.01	0.97
	0.92±0.01	0.93
	0.97±0.01	1.05
	0.91±0.01	
	0.93±0.01	
	0.93±0.01	
	m=0.94	m=0.98
C	2.59±0.02	2.75
	2.62±0.01	2.63
	2.61±0.02	2.55
	2.65±0.01	2.69
	2.71±0.02	
	2.61±0.01	
	m=2.63	m=2.66
D	0.21±0.01	0.19
	0.23±0.01	0.19
	0.22±0.01	0.20
		0.16
	m=0.22	m=0.19

a) Mean value with confidence limits at 95% levels (n=5, 5 scans). m: mean value of all data.

from the same aliquot solution for assay by the derivative method.

For practical purposes, the more convenient method I is recommended.

Four kinds of commercially obtained aspirin suppositories, A, B, C, and D were assayed, and the results are summarized in Table I (content of aspirin) and Table II (salicylic acid).

In the USP method, the hydrolysis of aspirin was unavoidable during the process of aspirin extraction by passing through the column. An examination of this column extraction technique using aspirin recrystallized from acetone clearly demonstrated the loss of aspirin and the increase of salicylic acid during this process. Therefore, it is not surprising that the values of aspirin content determined by the USP method in Table I are slightly lower than those found by the second derivative method. Nevertheless, the contents of aspirin found by the two methods showed a good agreement in all samples.

The nonuniformity of aspirin content between different aliquots of a sample was clearly seen in sample C. For this reason, the confidence limits of aspirin content were calculated for each aliquot. The results in Table I show the good reproducibility of the derivative method.

The results of salicylic acid assay in Table II also show a satisfactory agreement with the USP method, and a good reproducibility.

In conclusion, the assay of aspirin and salicylic acid in aspirin suppositories could be simply and rapidly performed by second derivative UV spectrometry without the need for troublesome extraction from suppository bases or separation of the pharmaceuticals.

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Salts of *myo*-Inositol Hexaphosphate in Alismatis Rhizoma and Angelicae Radix as an Indicator for Identification

NORIKO SHIMIZU and MASASHI TOMODA*

Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan

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Characteristic components useful as indicators for identification were isolated from Alismatis Rhizoma (rhizomes of *Alisma orientale* JUZEPCZUK or related species) and Angelicae Radix (roots of *Angelica acutiloba* KITAGAWA or related species). They were identified as sodium salts of *myo*-inositol hexaphosphate. A revision of the identification methods is proposed.

Keywords——*myo*-inositol hexaphosphate; Alismatis Rhizoma; *Alisma orientale*; Angelicae Radix; *Angelica acutiloba*; indication; identification method; electrophoresis; ^{13}C-NMR; gas chromatography

In previous reports, we proposed several methods for identification of Alismatis Rhizoma[1] and Angelicae Radix.[2] These methods included electrophoresis, paper partition chromatography (PPC), and thin-layer chromatography (TLC). Electrophoresis is particularly useful for identification owing to the presence of a characteristic component.

In this paper, we report the characterization of this component and a revision of the methods for identification of these crude drugs.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX270 FT NMR spectrometer in heavy water containing acetone as an internal standard at 30 °C. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector.

Materials——Alismatis Rhizoma was imported from China, and Angelicae Radix was prepared from *Angelica acutiloba* KITAGAWA cultivated in Nara prefecture. Crude drugs were purchased from Nakai-Kōshindo Co. in Kobe.

Isolation of the Components——The crude drug (50 g) was sliced and extracted twice with water (500 ml) under stirring in a boiling water bath for 30 min each time. After filtration, the filtrates were combined, and 5% cetyltrimethylammonium bromide (70 ml) was added to the filtrate (700 ml). The precipitate was separated by centrifugation, and dissolved in 0.5 M sodium chloride (70 ml). The solution was poured into two volumes of ethanol. The resulting precipitate was treated with 80% ethanol (140 ml), and after centrifugation, the final precipitate was dried *in vacuo*. The yields of this crude extract were 0.77 g from Alismatis Rhizoma and 1.20 g from Angelicae Radix.

The crude extract (0.77 g) from Alismatis Rhizoma was dissolved in water and applied to a column (5 × 76 cm) of Toyopearl HW 60. Elution was carried out with water, and fractions of 20 ml were collected and analyzed with a differential refractometer (Knauer No. 88.00). The eluates obtained from tubes 53 to 57 were combined, concentrated and applied to a column (5 × 84.5 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 46 were combined, concentrated and re-chromatographed on the same column of Sephadex G-25. The eluates obtained from tubes 43 to 45 were combined, concentrated and lyophilized. Substance Al (20 mg) was obtained as a white powder.

The crude extract (1 g) from Angelicae Radix was dissolved in water and applied to a column (5 × 86 cm) of Sephadex G-50. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 60 to 66 were combined, concentrated and applied to a column (5 × 83 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 36 to 47 were combined, concentrated and re-chromatographed on the same column of Sephadex G-25. The eluates obtained from tubes 38 to

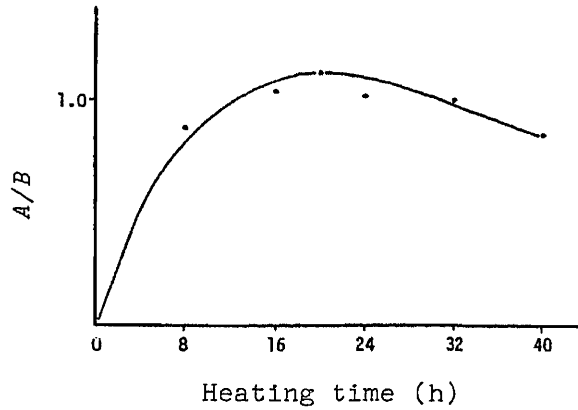

Fig. 1. The Influence of Heating Time in Acid Hydrolysis

A, *myo*-inositol liberated from phytic acid salt; *B*, *epi*-inositol (internal standard).

42 were combined, concentrated and lyophilized. Substance An (20 mg) was obtained as a white powder.

Electrophoresis, PPC and TLC——These were carried out under the same conditions as in a previous report.[1] Toluidine blue[3] was used for detection on electrophoresis and PPC, and the Hanes–Isherwood reagent[4] was used on TLC.

Acid Hydrolysis and Isolation of the Products——The sample (100 mg) was heated with 2 N hydrochloric acid (10 ml) at 125 °C for 20 h in a sealed tube. After removal of the acid by evaporation followed by addition of Dowex 2 (OH^-) to an aqueous solution of the residue, the filtrate and washing were combined, concentrated and applied to a column (5 × 82 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 60 to 62 were combined, concentrated and lyophilized.

GLC of the Hydrolysis Product——The sample (1 mg) was hydrolyzed with 2 N hydrochloric acid (1.5 ml) in a sealed tube at 125 °C, and the product was analyzed by GLC of inositol hexaacetates after various periods of time. The acid was removed from the hydrolysate by evaporation followed by repeated addition and evaporation of methanol. The residue was acetylated with acetic anhydride–pyridine mixture (1 : 1) at 100 °C for 1 h. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1 : 1) and subjected to GLC. GLC was performed on a column (3 mm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 225 °C with a helium flow of 60 ml per min. The retention times (min) of the hexaacetates of *myo*-, *scyllo*- and *epi*-inositols were 13.6, 17.2 and 21.5. For the determination of *myo*-inositol, *epi*-inositol was used as an internal standard. The influence of heating time is shown in Fig. 1.

Determination of Inorganic Components——Phosphorus was estimated by the method of Chen *et al.*[5] Sodium and magnesium were measured with a Shimadzu AA-610S atomic absorption/flame spectrophotometer.

Treatment with Phosphatase——The sample (50 mg) was dissolved in 0.05 M Tris–HCl buffer (pH 8.0) containing 0.001 M magnesium acetate (5 ml), and alkaline phosphatase (from calf intestine, Sigma Co., 25 mg) and three drops of toluene were added. The solution was incubated at 37 °C for 16 h, then the reaction mixture was successively passed through columns (0.7 × 5 cm) of Dowex 50WX8 (H^+) and Dowex 2 (OH^-). The eluate and washing with water were combined, concentrated and applied to a column (5 × 82.5 cm) of Sephadex G-25. Elution was carried out with water and fractions of 20 ml were collected. The eluates obtained from tubes 61 to 64 were combined and lyophilized. A part of the residue was acetylated and analyzed by GLC as described above.

Results and Discussion

The crude drugs were extracted with hot water. After filtration, the extract was treated with cetyltrimethylammonium bromide. The resulting precipitate was dissolved in sodium chloride solution and this solution was poured into ethanol. The precipitate obtained was dissolved in water, and applied successively to columns of Toyopearl HW60 and Sephadex G-25 in the case of Alismatis Rhizoma. For the isolation from Angelicae Radix, column chromatography was carried out with Sephadex G-50 and G-25 successively.

The characteristic substances (Al and An) obtained from Alismatis Rhizoma and Angelicae Radix, respectively, gave a single spot on electrophoresis, PPC and TLC. The two substances showed similar mobilities to each other in acidic buffer and mobile phase.[1]

The substances were hydrolyzed with 2 N hydrochloric acid at 125 °C for 20 h. The sole organic product was purified on a column of Sephadex G-25. The ^{13}C-NMR spectrum of the

TABLE I. Compositions of Substances Al and An and Calculated Values for Phytic Acid Salts

	myo-Inositol (%)	Phosphorus (%)	Sodium (%)	Magnesium (%)
Substance Al	24.9	24.6	8.4	2.5
Substance An	23.9	24.7	13.2	0.8
Phytic acid Na_3, Mg salt[a]	24.3	24.8	9.2	3.2
Phytic acid Na_5 salt[a]	23.6	24.1	14.9	—

a) Calculated.

purified product showed four signals at δ 73.765, 74.791, 75.033 and 76.949, and their integral ratio was *ca.* 2:1:2:1. By comparison of the ^{13}C-NMR spectrum with that of an authentic sample, the product was identified as *myo*-inositol.

The purified product was acetylated and analyzed by GLC. *myo*-Inositol hexaacetate was identified by GLC, and it was clearly distinguishable from hexaacetates of *epi*- and *scyllo*-inositols. The substances Al and An also produced *myo*-inositol on treatment with an alkaline phosphatase, and the identity of the product was confirmed by GLC of its acetate.

The ^{13}C-NMR spectra of the substances Al and An each showed four signals at δ 76.002, 77.917, 78.754 and 80.157, and δ 75.894, 77.890, 78.565 and 79.995, respectively. Their integral ratio was *ca.* 2:1:2:1 in both cases. The three-sodium salt of *myo*-inositol hexaphosphate (phytic acid) prepared from phytin gave a ^{13}C-NMR spectrum having four signals at δ 75.840, 77.890, 78.538 and 79.995 in the integral ratio of *ca.* 2:1:2:1.

Quantitative analyses of the constituents of the substances Al and An gave the values shown in Table I. The calculated values for the three-sodium and one-magnesium salt and five-sodium salt of *myo*-inositol hexaphosphate are also given in Table I.

Based on the accumulated evidence described above, it can be concluded that substances Al and An are both sodium salts of *myo*-inositol hexaphosphate. They are partly combined with magnesium, but the kind and the content of cations are not important from the viewpoint of the identification of crude drugs. No calcium was found in the substances Al and An. In a previous report,[1] we described the appearance of lactose phosphate in Alismatis Rhizoma, but it was a contaminant from a commercial enzyme preparation.

Phytin is widely distributed in various parts of higher plants, although it is insoluble in water. We have already found that the hot water extracts of many crude drugs gave a positive spot test in the color reaction with toluidine blue.[1,2] After studies on one hundred and three kinds of crude drugs, which are generally used in oriental pharmaceutical preparations, by means of this spot test, electrophoresis and GLC of the acetates of acid hydrolysates, twenty-three kinds of samples were found to be *myo*-inositol phosphate-containing crude drugs. They are Alismatis Rhizoma, Angelicae Radix, Angelicae Dahuricae Radix, Arisaemae Tuber, Armeniacae Semen, Bupleuri Radix, Curcumae Rhizoma, Cyperi Rhizoma, Dioscoreae Rhizoma, Dolichi Semen, Glehniae Radix cum Rhizoma, Houttuyniae Herba, Lilii Bulbus, Mori Cortex, Moutan Cortex, Nelumbi Fructus, Paeoniae Radix, Persicae Semen, Platycodi Radix, Peucedani Radix, Saposhnikoviae Radix, Scrophulariae Radix, and Trichosanthis Semen.

In order to find which crude drugs contain phytic acid salts in abundance, the hot water extracts of these twenty-three crude drugs were subjected to precipitation procedures under the same conditions as in a previous report,[1] except that the final dried precipitates, obtained

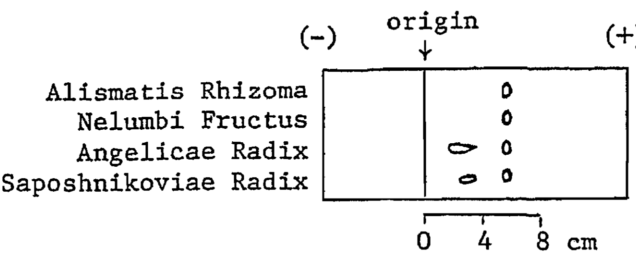

Fig. 2. Cellulose Acetate Membrane Electrophoresis with Pyridine–Acetic Acid Buffer

from one gram of each sample, were dissolved in 10 ml of water. Each of the resulting solutions (0.5 μl) was used as a sample for electrophoresis. Only four crude drugs, Alismatis Rhizoma, Angelicae Radix, Nelumbi Fructus, and Saposhnikoviae Radix, gave clear positive spots with toluidine blue under these conditions. Alismatis Rhizoma and Nelumbi Fructus gave a single spot of *myo*-inositol hexaphosphate. On the other hand, Saposhnikoviae Radix showed additional tailing in the electrophoretic pattern, probably due to pectic substances,[6] as also did Angelicae Radix (Fig. 2).

As already reported in previous papers,[1,2] three samples of Alismatis Rhizoma, *i.e.*, fresh rhizomes of *Alisma orientale* and crude drugs from Ssuchuan and Fuchien, China, showed the same reaction. In the case of Angelicae Radix, crude drugs and fresh roots of *Angelica acutiloba* and of *Angelica acutiloba* KITAGAWA var. *sugiyamae* HIKINO also gave the same result in the identification tests.

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Construction of a *p*-Nitrophenolate-Sensitive Membrane Electrode and Its Application to an Enzyme Assay

TAKASHI KATSU,* MIEKO KANAMITSU, and YUZABURO FUJITA

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

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A *p*-nitrophenolate-sensitive membrane electrode was constructed by using tetraheptylammonium chloride as an ion-exchanger and *o*-nitrophenyl phenyl ether as a membrane solvent. The electrode responded to *p*-nitrophenolate down to 1 μM in a buffer solution of 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10). The selectivity coefficients of the electrode to typical anions increased in the following order: $Cl^- < NO_3^- < SCN^- \approx$ *p*-nitrophenolate $< ClO_4^-$. The method was applied to determine the enzyme activity of alkaline phosphatase.

Keywords——*p*-nitrophenolate; *p*-nitrophenolate-sensitive electrode; ion-selective electrode; membrane electrode; enzyme assay; alkaline phosphatase; electrochemical analysis

Many *p*-nitrophenol derivatives have been prepared and utilized as enzyme substrates.[1] The *p*-nitrophenol released by the enzymatic cleavage of the substrate, giving a yellow color in alkaline solution, is usually determined by colorimetry.[2,3] Here, we report another method for detecting *p*-nitrophenolate by using a *p*-nitrophenolate-sensitive membrane electrode. This electrochemical analysis using a selective ion-sensitive electode has the inhrent advantage of being simple, rapid, and continuous.[4] The present method provides a new technique for enzyme assay.

Materials and Methods

A *p*-nitrophenolate-sensitive membrane electrode was constructed by the use of a poly(vinyl chloride)-based membrane.[4] The components of the membrane were: 0.5 mg of tetraheptylammonium chloride, 60 μl of *o*-nitrophenyl phenyl ether, and 30 mg of poly(vinyl chloride). These materials were dissolved in 1—2 ml of tetrahydrofuran. The solution was poured into a flat Petri dish of 30 mm in diameter, and then the solvent was evaporated at room temperature. The resulting membrane was cut and stuck on a poly(vinyl chloride) tube with tetrahydrofuran. The membrane was conditioned overnight in a solution containing 1 mM *p*-nitrophenol and 10 mM Na_2CO_3–$NaHCO_3$ (pH 10). The electrochemical cell arrangement was as follows: Ag, AgCl/1 M KCl/internal solution/sensor membrane/sample solution/1 M KCl/Ag, AgCl. The internal solution was the same as the one used in the conditioning. The selectivity coefficients of the electrode were determined by the separate solution method[5] in 10 mM solutions of the respective sodium salts dissolved in 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10).

Alkaline phosphatase (ALP) was purchased from Sigma Chemical Co. (product number P5521; lot number 95F-8220-1). This enzyme had the activity of 938 units/ml [determined in a buffer solution containing 0.1 M glycine (pH 10.4), 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 6 mM *p*-nitrophenyl phosphate at 37 °C]. One unit was defined as the amount of the enzyme hydrolyzing 1 μmol of *p*-nitrophenyl phosphate per min under the above conditions.

Results and Discussion

Up to the present, *p*-nitrophenolate has been determined colorimetrically by measuring the absorbance around 400—420 nm.[2,3] We measured the absorbance at 405 nm and obtained a value of 0.18 at the concentration of 10 μM *p*-nitrophenolate in a buffer solution of 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10). This indicates that *p*-nitrophenolate can easily be

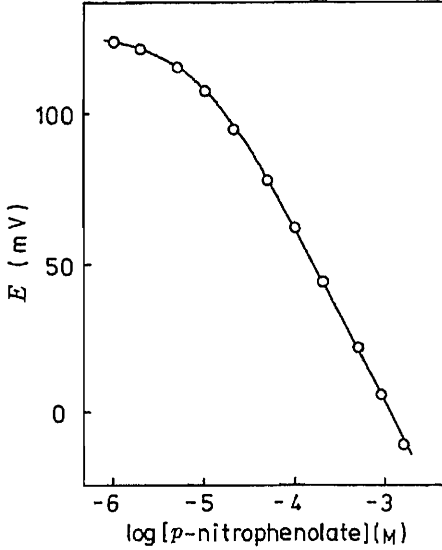

Fig. 1. Response of a *p*-Nitrophenolate-Sensitive Electrode in 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10) at 28 °C

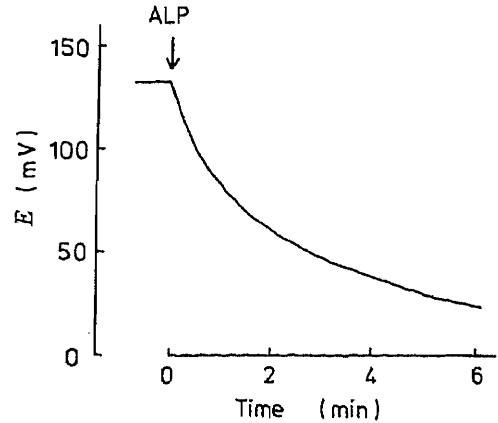

Fig. 2. Monitoring of *p*-Nitrophenolate Released by the Enzymatic Cleavage of *p*-Nitrophenyl Phosphate Catalyzed by ALP

Commercially available enzyme suspension (Sigma, P5521) was diluted 10 times. At the arrow, 2.5 μl of the diluted enzyme suspension was added to 1 ml of a buffer solution comprising 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10), 2 mM $MgCl_2$, and 6 mM *p*-nitrophenyl phosphate. Measurements were made at 28 °C. The enzyme had the activity of 0.23 unit under the conditions described in Materials and Methods.

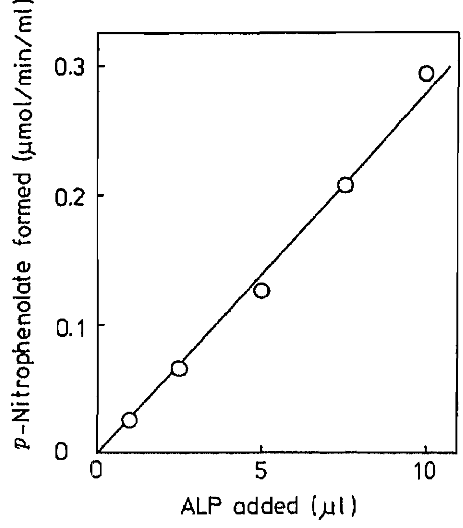

Fig. 3. The Correlation between the Amounts of ALP and of *p*-Nitrophenolate Formed

Enzymatic reaction was performed under the same conditions as in Fig. 2, except that the amount of ALP was changed as indicated.

determined by the colorimetric method down to concentrations of around 1—10 μM: Figure 1 shows the calibration curve of the present electrode measured in the same buffer solution of 0.1 M Na_2CO_3–$NaHCO_3$ (pH 10). The electrode responded to *p*-nitrophenolate down to 1 μM. This detection limit is comparable to that of the colorimetric method, though the sensitivity of the electrode decreased in the lower concentration range. It should be emphasized, however, that even at lower concentrations, *p*-nitrophenolate can be determined with the appropriate calibration. The slope of the electrode response in the linear range was −58 mV/decade. The response time of the electrode was less than 10 s when the concentration of *p*-nitrophenolate was changed from 10 to 100 μM. The selectivity coefficients, log $K^{\mathrm{Pot}}_{p\text{-NP},X}$, were: Cl^-, −3.5; benzoate, −2.9; benzenesulfonate, −1.8; NO_3^-, −1.8; *o*-nitrophenolate, −1.0; SCN^-, −0.1; ClO_4^-, 0.8; 2,4-dinitrophenolate, 1.3; picrate, 3.2. As is clear from the sequence of the selectivity coefficients, the responses of the present electrode to many lipophilic anions were

stronger than that to *p*-nitrophenolate. The interference from lipophilic anions is characteristic of an ion-selective electrode prepared with an ion-exchanger.[6] Since anions showing stronger response than *p*-nitrophenolate were not contained in usual biological fluids, we tried to apply the present electrode to an enzyme assay. Figure 2 shows the detection of *p*-nitrophenolate formed by the enzymatic reaction of ALP and *p*-nitrophenyl phosphate. It was confirmed that the electrode did not respond to substrate, enzyme, or phosphate coexisting in the reaction mixture. We further measured the relation between the amounts of ALP and of *p*-nitrophenolate formed, and obtained a good correlation (Fig. 3).

These results demonstrate clearly that the *p*-nitrophenolate-sensitive electrode can be applied for the determination of the enzyme activity. The present method represents a new basic technique for enzyme assay and would also be applicable to a recently-developed enzyme immunoassay[7] using *p*-nitrophenol derivatives as substrates.

Acknowledgment We are grateful to Drs. Norio Koide of Okayama University Medical School and Takashi Hirota of this faculty for valuable discussions.

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Effect of Dietary Essential Fatty Acids on Pulmonary Metastasis of Ascites Tumor Cells in Rats[1)]

TAKAMITSU HORI,[a] ATSUKO MORIUCHI,[a] HARUMI OKUYAMA*,[a]
TAKESHI SOBAJIMA,[b] KEIKO TAMIYA-KOIZUMI,[b]
and KIYOHIDE KOJIMA[b]

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University,[a] Tanabedori, Mizuho-ku, Nagoya 467, Japan and Laboratory of Cancer Cell Biology, Nagoya University School of Medicine,[b] Tsurumai, Showa-ku, Nagoya 466, Japan

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Rats were fed a semi-purified diet supplemented either with a linoleic acid-rich oil (safflower oil) or with an α-linolenic acid-rich oil (perilla oil) from weanling to 7 weeks of age. Rats fed a conventional diet were also used as controls. Ascites tumor cells were injected intravenously into these rats and the numbers of metastatic foci on the pulmonary surface were determined macroscopically. The average number of metastatic foci was significantly less in the rats fed the perilla oil diet than in the rats fed the safflower oil diet or the conventional diet. Combined with previous results on the metastatic potentials of tumor cells modified with different fatty acids, these results indicate that the modification of host animal cells by dietary fatty acids also affects the metastatic potentials of the ascites tumor cells in rats, apparently independently of the tumor cell modifications caused by the supplemented fatty acids.

Keywords——metastasis; tumor; linoleate; α-linolenate; platelet; diet; rat; fatty acid composition

Epidemiologically, mammary tumorigenesis has been positively correlated with the amount of fat intake.[2,3)] Although polyunsaturated fatty acids were once supposed to lead to an increase in tumorigenesis through the action of lipid peroxide,[4-7)] it was later shown that the intake of higher amounts of saturated fats also increased the incidence of mammary tumor in animals.[8)] Polyunsaturated fatty acids in animal tissues are classified mainly into the n-6 series derived from linoleic acid (18 : 2 n-6) and then n-3 series derived from α-linolenic acid (18 : 3 n-3). The essentiality of linoleic acid has been established and its physiological functions appear to be mediated through the hormone-like eicosanoids derived from arachidonic acid (20 : 4 n-6). Some eicosanoids from arachidonate are inhibitory for tumor cell growth while others appear to be stimulatory,[9-11)] indicating the complicated involvement of arachidonate in the division, growth and differentiation of tumor cells. As to the metastasis of tumor cells, the administration of prostaglandin I_2 (PGI_2) or a drug inducing an increase in PGI_2 inhibited the metastasis of tumor cells, leading to the hypothesis that platelet–tumor cell interaction is important in the metastatic phenomena.[12-14)]

In contrast to the n-6 series fatty acids, the essentiality of α-linolenic acid for higher animals had been questioned,[15)] until we recently showed that it is essential for maintaining proper learning ability in rats.[16)] Eicosanoids appear to be synthesized also from an n-3 fatty acid, eicosapentaenoic acid (20 : 5 n-3), but only to a limited extent. Their physiological activities are generally lower than those of the eicosanoids from n-6 fatty acids, and no specific functions have been ascribed to the eicosanoids from n-3 acids. However, n-3 polyunsaturated fatty acids in general can compete with n-6 fatty acids[17,18)] and regulate their metabolism in

animal tissues.[19] One of the consequences of increasing n-3 fatty acids in the diet is supposed to be a decrease in platelet aggregability and thrombotic tendency.[20,21] The decrease in platelet aggregability may decrease the metastatic potentials of tumor cells through platelet–tumor cell interaction.[13] To test this possibility, we examined the metastatic potentials of ascites sarcoma cells in rats fed either with a linoleate-rich diet or with an α-linolenate-rich diet.

Materials and Methods

Diets——The basic components of a semi-purified diet (Nippon Clea Corp., Tokyo) were 24.6% milk casein, 47.0% corn starch, 2.0% α-starch, 8.0% cellulose, 5.0% sucrose, 2.0% vitamin mixture, 6.0% minerals, 0.4% methionine, and 5.0% oil (safflower oil or perilla oil). A conventional diet (Nippon Clea, CE-2) was also used as a control. Fatty acid compositions were 10.6% saturated, 12.3% oleic, 12.8% linoleic and 64.0% α-linolenic acids in the perilla oil diet, 11.3% saturated, 10.4% oleic, 78.0% linoleic and 0.05% α-linolenic acids in the safflower oil diet, and 18.3% saturated, 22.2% oleic, 49.7% linoleic and 4.1% α-linolenic acids in the normal diet; the details are described elsewhere.[16]

Animals——Donryu strain rats at 3 weeks of age were purchased and fed the test diets for 4 weeks prior to use in the experiment on metastasis.

Assay of Metastatic Potential——Yoshida sarcoma cells were maintained by intraperitoneal transplantation in inbred Donryu strain rats. A suspension of 10^4 cells was injected into the iliac vein of a male Donryu rat at 5 weeks of age. After 2 weeks, the number of metastatic foci on the lung surface was determined macroscopically.[22]

Results and Discussion

The number of pulmonary metastatic foci was significantly less in the perilla group than in the safflower group, and also significantly less than in the normal diet group. No statistically significant difference was observed between the safflower group and the normal diet group. The difference in metastatic foci between the perilla group and the safflower group was statistically significant ($p<0.05$) (Table I). Two other experiments with rats fed the perilla oil diet and safflower oil diet through two generations showed similar results; the metastatic foci in the perilla group amounted to 60% of those in the safflower group (data not shown).

The difference in the fatty acid compositions of diets was reflected in the fatty acid compositions of plasma lipids and tissue lipids; these results have been reported in part elsewhere.[16,23] Generally, the proportions of 20:5 n-3 and 22:6 n-3 were higher and those of 18:2 n-6 and 20:4 n-6 were lower in the perilla group as compared with the safflower group. In the platelet phospholipids, the 20:5 n-3/20:4 n-6 ratios were 0.826 and 0.002 in the perilla group and safflower group, respectively.

Previously, we showed that the tumor cells supplemented with 20:5 n-3 or 22:6 n-3 exhibited higher stickiness to a glass surface, lower ability to pass through capillary vessels and a larger number of metastatic foci as compared with controls.[22] In contrast to those results, the metastatic potential of the tumor cells was less in the rats enriched with 20:5 n-3 (Table I). Thus, not only the modification of tumor cells but also the modification of host

TABLE I. Effects of Dietary Fat on Pulmonary Metastasis

Dietary fat	Number of metastatic foci per rat[a]	Relative value[a]
Safflower oil diet	7.0±1.3 (10)	100
Perilla oil diet	3.8±0.6[b] (10)	54±6[b]
Normal diet	8.8±0.1 (10)	126±1

a) Values are means±S.E. Numbers in parentheses indicate the numbers of rats used. b) Statistically significant differences between the safflower oil group and the perilla oil group ($p<0.05$ in Student's t-test).

animal cells with different fatty acids had a significant influence on the metastatic potential of the tumor cells. The enrichment of n-3 fatty acids in the host cells and tumor cells affected metastasis differently.

The mechanisms inducing such a difference in the metastatic potential of tumor cells can not be easily explained. Since the membrane lipid acyl chains of endothelial cells are known to be modified by dietary fatty acids, eicosanoids formed in these cells are probably different, and might have affected the metastatic potential of tumor cells. Possible differences in platelet aggregability, expected from the changes in platelet fatty acids, might also affect the metastasis.

The present results, however, may have an etiological and practical significance, since one of the major dietary changes in the past 30 years in Japan has been characterized as a decrease in the n-3/n-6 ratio of the dietary fats (data not shown). Although the animal model used in the present experiments may be only partially relevant to the actual metastasis of syngenic tumors, the supplementation of n-3 series fatty acids may have a preventive effect on metastasis of tumor cells in humans. In this context, it seems interesting to note that tumorigenesis[5] and tumor growth[24] are also inhibited by dietary n-3 fatty acids in animal models.

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A New Antiarrhythmic Peptide, *N*-3-(4-Hydroxyphenyl)propionyl Pro–Hyp–Gly–Ala–Gly

YASUHIRO KOHAMA,*,[a] NAOTSUGU OKIMOTO,[a] TSUTOMU MIMURA,[a] CHIKARA FUKAYA,[b] MASAHIRO WATANABE[b] and KAZUMASA YOKOYAMA[b]

Faculty of Pharmaceutical Sciences, Osaka University,[a] Yamadaoka 1-6, Suita, Osaka 565, Japan and Central Research Laboratories, Green Cross Corporation, Ltd.,[b] Chuo-1, Johto-ku, Osaka 532, Japan

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In order to increase the antiarrhythmic activity of Pro–Hyp–Gly–Ala–Gly (P-5), P-5 analogues with three different hydrophobic substituents, *N*-3-(4-hydroxyphenyl)propionyl (H), *N*-3-phenylpropionyl (I) and *N*-3-phenylpropyl (P), were prepared and their activities were evaluated in $CaCl_2$-induced arrhythmias in mice. HP-5 showed potent antiarrhythmic activity at 1 mg/kg, i.v. and its potency was much higher than that of P-5 at 10 mg/kg, i.v. IP-5 showed similar potency to P-5, but PP-5 was inactive. Pro–Hyp–Gly–Ala, Pro–Hyp–Gly and Pro–Hyp with the substituent, H, were also ineffective. Thus, 3-(4-hydroxyphenyl)propionylation of the imino nitrogen of Pro in P-5 led to increased potency.

Keywords——peptide analogue; antiarrhythmic effect; $CaCl_2$ arrhythmias; mouse

A hexapeptide (antiarrhythmic peptide, AAP), isolated from bovine atria[1] and identified as Gly–Pro–Hyp–Gly–Ala–Gly,[2] showed a protective effect against experimental drug-induced arrhythmias in cultured myocardial cells of rats[1] and whole hearts of dogs, rats and mice.[3] Although several investigations have been done on the structure–activity relationship of AAP analogues including its fragments and collagen peptides, all the tested analogues were ineffective.[1,2,4] In this paper, potent antiarrhythmic activity of a new analogue, Pro–Hyp–Gly–Ala–Gly with a hydrophobic substituent at its amino-terminus, is demonstrated.

Experimental

Analogues——The peptide analogues, listed in Table I, were synthesized by the dicyclohexylcarbodiimide method.[2,5] Briefly, Pro–Hyp(OBzl)–Gly–Ala–Gly·OBzl was coupled with 3-(4-benzyloxyphenyl)propionic acid or 3-phenylpropionic acid, and protecting groups were removed by catalytic reduction to give HP-5 or IP-5, respectively. PP-5 was synthesized by coupling Hyp(OBzl)–Gly–Ala–Gly·OBzl with *N*-3-phenylpropyl Pro. The structural proofs of analogues were based on proton nuclear magnetic resonance (^1H-NMR) spectroscopy and amino acid analysis (Table I). The analogues dissolved in saline and adjusted to pH 7.4 were used throughout experiments. Quinidine sulfate (Nakarai Chemicals Ltd.) was used as a reference drug.

Measurement of Antiarrhythmic Activity——According to the method of Lynch *et al.*,[6] male ddY mice weighing 25—35 g, were anesthetized with sodium pentobarbital (30—40 mg/kg, i.p.) and given $CaCl_2$ (3 mg/0.1 ml/25 g body weight/min) into the right femoral vein by slow infusion. Test samples were given into the left femoral vein 3 min before the $CaCl_2$ infusion was started. The lead II ECG was continuously recorded on a Polygraph system RM-6000 (Nihon Kohden). The times to onset of arrhythmias (atrioventricular block of 2nd or 3rd degree, or ectopic beats) were measured and compared in animals pretreated with saline (control group) and test samples. Prolongation (%) of the onset time of arrhythmias was considered as the criterion of the protective effect of test samples.[7]

TABLE I. Peptide Analogues

	Analogue	Amino acid ratios[a]				^{1}H-NMR[b] δ
		Hyp	Pro	Gly	Ala	
P-5	Pro–Hyp–Gly–Ala–Gly	1.02	0.97	2.00*	0.97	N.D.[c]
P-4	Pro–Hyp–Gly–Ala	0.91	0.99	1.00*	0.98	1.31 (d, 3H, J=7 Hz), 1.9—2.6 (m, 6H), 3.3—3.5 (m, 2H), 3.6—3.8 (m, 3H), 4.0—4.1 (m, 1H), 4.2—5.7 (m, 5H)
P-3	Pro–Hyp–Gly	0.93	1.00	1.00*		1.8—2.5 (m, 6H), 3.2—3.8 (m, 6H), 4.4—4.7 (m, 3H)
P-2	Pro–Hyp	0.97	1.00**			1.9—2.5 (m, 6H), 3.35—3.45 (m, 2H), 3.55—3.7 (m, 2H), 4.3—4.6 (m, 3H)
HP-5	*N*-3-(4-Hydroxyphenyl)-propionyl P-5	1.02	0.99	2.00*	0.96	1.38 (d, 3H, J=7 Hz), 1.7—2.4 (m, 6H), 2.4—3.0 (m, 4H), 3.1—4.1 (m, 9H), 4.2—4.9 (m, 3H), 6.65 (d, 2H, J=8 Hz), 6.97 (d, 2H, J=8 Hz)
IP-5	*N*-3-Phenylpropionyl P-5	0.97	1.01	2.00*	0.99	1.38 (d, 3H, J=7 Hz), 1.5—3.1 (m, 10H), 3.1—4.8 (m, 12H), 7.12 (s, 5H), 7.5—8.7 (m, 3H)
PP-5	*N*-3-Phenylpropyl P-5	0.99		2.00*	0.97	1.26 (d, 3H, J=7 Hz), 1.5—2.3 (m, 11H), 2.3—2.9 (m, 4H), 3.0—4.0 (m, 9H), 4.0—4.6 (m, 3H), 5.8—6.5 (s, 4H), 7.12 (s, 5H), 7.7—8.0 (m, 1H), 8.2—8.6 (m, 1H)
HP-4	*N*-3-(4-Hydroxyphenyl)-propionyl P-4	0.99	1.01	1.00*	1.07	1.3—1.4 (m, 3H), 1.7—2.4 (m, 6H), 2.4—2.7 (m, 2H), 2.7—2.9 (m, 2H), 3.5—4.1 (m, 6H), 4.2—4.4 (m, 1H), 4.54—4.7 (m, 3H), 6.6—6.8 (m, 2H), 6.9—7.1 (m, 2H)
HP-3	*N*-3-(4-Hydroxyphenyl)-propionyl P-3	0.93	1.02	1.00*		1.8—2.4 (m, 6H), 2.4—2.7 (m, 2H), 2.7—2.9 (m, 2H), 3.3—3.9 (m, 8H), 4.35—4.7 (m, 3H), 6.69 (d, 2H, J=8 Hz), 7.03 (d, 2H, J=8 Hz)
HP-2	*N*-3-(4-Hydroxyphenyl)-propionyl P-2	0.96	1.00**			1.75—2.4 (m, 6H), 2.5—2.9 (m, 4H), 3.35—3.75 (m, 4H), 4.25—4.6 (m, 3H), 6.6—6.75 (m, 2H), 6.95—7.1 (m, 2H)

a) Amino acid analysis was performed on a Hitachi 034 liquid chromatograph for 6 N HCl hydrolysates at 110 °C for 24 h (recovery of Gly* or Pro**, 90% or 84%). *b*) ^{1}H-NMR spectra were measured with a Hitachi R-24B (60 MHz) or a Bruker AC-200 (200 MHz) spectrometer in MeOH-d_4 (except for HP-5 in MeOH-d_4+$CDCl_3$, and IP-5 and PP-5 in dimethyl sulfoxide-d_6+$CDCl_3$) with tetramethylsilane as an internal standard. *c*) Not determined.

Results and Discussion

We have reported that the AAP analogues including Gly–Pro, Gly–Pro–Hyp, Gly–Pro–Leu, (Pro–Pro–Gly)$_5$, Gly–Pro–Hyp–Gly, Gly–Pro–Leu–Gly–Pro, Hyp–Gly–Ala–Gly, Gly–Ala–Gly, Gly–Gly–Gly and 9 kinds of collagen peptides, whose sequences differ by only one amino acid from that of AAP, showed no antiarrhythmic activity.[1,2,4] Among newly synthesized fragments including P-5, P-4, P-3 and P-2, only P-5 showed almost the same antiarrhythmic activity at 10 mg/kg, i.v. as AAP and quinidine sulfate in $CaCl_2$-induced arrhythmias in mice, as shown in Table II. Therefore, it is probable that the penta amino acid sequence of P-5 is the minimum essential requirement for the antiarrhythmic activity of the peptide. However, intravenously administered P-5 might show low activity because of the high hydrophilicity of the chemical structure. Thus, in order to increase the activity of P-5, P-5 analogues with three different hydrophobic substituents, *N*-3-(4-hydroxyphenyl)propionyl, *N*-3-phenylpropionyl and *N*-3-phenylpropyl, were prepared and their antiarrhythmic activities were evaluated (Table II). HP-5 showed potent antiarrhythmic activity at 1 and 10 mg/kg, i.v.

Table II. Effect of Peptide Analogues on $CaCl_2$-Induced Arrhythmias in Mice

Sample[a]	Dose (mg/kg)	No. of mice	Antiarrhythmic activity[b]	
			Onset of arrhythmia (s, mean ± S.E.)	Prolongation (%)
Control	—	32	96 ± 6	—
AAP	1	8	98 ± 8	2
	10	8	129 ± 12[c]	34
P-5	1	8	95 ± 11	0
	10	8	128 ± 7[c]	33
P-4	10	8	113 ± 13	18
P-3	10	8	106 ± 9	10
P-2	10	8	112 ± 11	17
Quinidine	1	8	93 ± 10	0
sulfate	10	8	127 ± 10[c]	32
Control	—	44	89 ± 5	—
HP-5	0.1	8	107 ± 6	20
	1	8	144 ± 12[d]	62
	10	8	166 ± 19[d]	87
IP-5	1	8	110 ± 5	24
	10	8	123 ± 16[c]	38
PP-5	1	8	80 ± 12	0
	10	8	81 ± 8	0
HP-4	10	8	97 ± 5	9
HP-3	10	8	89 ± 9	0
HP-2	10	8	103 ± 5	16

a) Sample or saline (control) was administered intravenously 3 min before the start of $CaCl_2$ infusion (3 mg/0.1 ml/25 g body weight/min). *b*) Onset time of arrhythmias induced by $CaCl_2$ infusion was measured. See Experimental for details. *c*) $p<0.05$, *d*) $p<0.001$: *versus* control.

and its protective effect (prolongation) was much higher than that of P-5. IP-5 showed similar potency to P-5, but PP-5 was inactive. As regards the structure–activity relationship, acylation (HP-5 and IP-5) of the imino nitrogen of Pro in P-5 was necessary for the activity, because alkylation (PP-5) of the imino nitrogen reduced the activity. Further, hydroxylation of the phenyl group was most effective, because HP-5 was much more potent than IP-5. On the other hand, peptide fragments with the *N*-3-(4-hydroxyphenyl)propionyl group, HP-4, HP-3 and HP-2, were ineffective, as shown in Table II. Therefore, the potent antiarrhythmic activity of HP-5 was not due only to the substituent. Thus, 3-(4-hydroxyphenyl)propionylation of the imino nitrogen of Pro in P-5 led to increased potency.

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Antifungal and Insecticidal Activities of Daphnodorins A, B and C

YOSHIHIKO INAMORI,* KAZUO TAKEUCHI, KIMIYE BABA
and MITSUGI KOZAWA

Osaka University of Pharmaceutical Sciences, Kawai, 2-10-65, Matsubara-shi, Osaka 580, Japan

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Three flavans, daphnodorin A (**1**), daphnodorin B (**2**) and daphnodorin C (**3**), isolated from the root and the bark of *Daphne odora* THUNB., showed antifungal and insecticidal activities. Of these compounds, **1** and **3** showed rather strong antifungal activities against *Pyricularia oryzae*. The protective values (%) of **1** and **3** were 58 and 40 at the low concentration of 10 ppm, respectively. On the other hand, compound **2** exhibited weak insecticidal activity against *Spodoptera litura*, *Callosobruchus chinensis* and *Tetranychus urticae*. The insecticidal activities of **1** and **3** were weaker than that of **2**.

Keywords——daphnodorin A; daphnodorin B; daphnodorin C; flavan; *Daphne odora*; Thymelaeceae; insecticidal activity; antifungal activity; *Pyricularia oryzae*

We recently isolated three new flavans, daphnodorin A (**1**), daphnodorin B (**2**) and daphnodorin C (**3**), together with four known coumarins, daphnetin, daphnoretin, umbelliferone and daphneticin, from the root and bark of *Daphne odora* THUNB.[1] It had already been reported that diterpenes and their esters from this plant show several biological activities including nematicidal activity.[2]

As compounds **1**—**3** have unique structures, we have examined their biological activities. In this paper, we report their antifungal and insecticidal activities.

HO OH HO OH OH OH OH OH OH OH OH OH OH HO HO HO O O O O O O O O O O

1 2 3

Chart 1

Materials and Methods

Chemicals——Daphnodorin A (**1**), daphnodorin B (**2**) and daphnodorin C (**3**) were used. These compounds have already been isolated from the root and bark of *Daphne odora* THUNB. by the authors.[1]

Organisms——The plant pathogenic fungi used were as follows: *Pyricularia oryzae*, *Rhizoctonia solani*, *Phytophora infestans*, *Botrytis cinerea*, *Puccinia recondita* and *Erysiphe graminis*. The host plants were *Oriza sativa* L., *Lycopersicon esculentum* MILL., *Pisum sativum* L. var. *arvense* POIL. and *Triticum aestivum* L. The insects used were as follows: *Spodoptera litura* FABRICIUS (3rd instar larvae), *Nilaparvata lugens* STAL (3rd instar larvae) *Callosobruchus chinensis* L. (adult) and *Tetranychus urticae* KOCH (adult and egg).

Biological Activity Test——1) The First Antifungal Screening Test: Suspensions of spores and mycelia of each of the plant pathogenic fungi were inoculated onto leaves of the corresponding host plants spread with chemicals. The treated leaves were incubated for 5 d in a hothouse. The numbers of fungi that grew on the leaves were counted with the naked eye. The protective value (%) was determined from the numbers of fungi in the treated and control groups. Any sample with a protective value above 90% was further examined in secondary screening tests.

2) The Second Antifungal Screening Test on *P. oryzae*: i) The protective effect: the tests were carried out at low concentrations of 10, 50 and 200 ppm, respectively, by the same method as used for the first screening test, and ii) the residual effect (%) on leaves: after 3 d of chemical treatment, leaves of rice plant (*Oriza sativa*) were inoculated with suspensions of spores of *P. oryzae*. The treated leaves were incubated for 5 d in a hothouse. The numbers of fungi that grew on the leaves were counted with the naked eye.

3) Insecticidal Activity Tests: i) Leaf and stem dipping: for *S. litura*, toxicity was examined by releasing the larva onto leaves treated with the chemicals. Leaves of cabbage plants were dipped for 5 s into an aqueous solution of a test chemical, then dried in air, and fed to the larva in Petri dishes. For *N. lugens*, the toxicity of chemicals applied to the stem of rice plant (*Oriza sativa*) was examined. The stems were dipped for 5 s into a test solution, then dried in air, and placed in test tubes into which the insects were released. For *T. urticae*, the toxicity of chemicals applied to the leaves of Ingenmame (*Phaseolus vulgaris* L.) was examined. The leaves were dipped for 5 s into the test solutions, then dried in air, and placed in Petri dishes into which insects bearing eggs were released. ii) Spraying method: test solutions were sprayed onto *C. clinensis* with a sprayer. Both bait, adzuki beans (*Phaseolus angularis*) and water were available to the insects. The insect mortalities in all experiments were counted for 5 d after the insect release. The experimental size was 50 insects/group and 2 groups/chemical.

Temperature——Every experiment was carried out at 25—27 °C.

Results

Antifungal Activities of Daphnodorins A (1), B (2) and C (3) on Plant-Pathogenic Fungi

The antifungal activities of compounds **1**, **2** and **3** on plant pathogenic fungi were examined. The results are summarized in Tables I and II. In the first antifungal screening test, compounds **1** and **3** showed rather strong antifungal activities against *Pyricularia oryzae*; the protective values (%) of **1** and **3** were 95 and 90, respectively, at the concentration of 500 ppm. On the other hand, the antifungal activity of **2** on *P. oryzae* was weaker than those of **1** and **3**. Compounds **1**—**3** did not show antifungal activities against plant-pathogenic fungi other than *P. oryzae*. Because compounds **1** and **3** showed protective values above 90%, both compounds were further examined in the second antifungal screening test.

In the second antifungal screening test, compounds **1** and **3** showed protective values (%) of 58 and 40, respectively, against *P. oryzae* even at the low concentration of 10 ppm. Further, compounds **1** and **3** showed residual effects (%) of 48 and 40, respectively, on leaves at the concentration of 500 ppm.

TABLE I. Antifungal Activities of Daphnodorins A (1), B (2) and C (3) on Plant-Pathogenic Fungi in the First Screening Test

Fungi	Host plant	Protective value (%)		
		1	2	3
Pyricularia oryzae	*Oriza sativa* L.	95	79	90
Rhizoctonia solani	*Oriza sativa* L.	0	0	0
Phytophthora infestans	*Lycopersicon esculentum* MILL.	0	0	0
Botrytis cinerea	*Pisum sativum* L. var. *arvense* POIL.	0	0	0
Puccinia recondita	*Triticum aestivum* L.	0	0	0
Erysiphe graminis	*Triticum aestivum* L.	0	0	0

Culture conditions: 27 °C, 5 d. Concentration: 500 ppm. Method: spraying method.

TABLE II. Antifungal Activities of Daphnodorin A (1) and C (3) on *Pyricularia oryzae* in the Second Screening Test

Concentration (ppm)	Protective value (%)		Residual effect (%)[a]	
	1	**3**	**1**	**3**
200	93	89		
50	83	69		
10	58	40		
500			48	40

Culture conditions: 27 °C, 5 d. Host plant: *Oriza sativa*. Method: spraying method. *a*) Residual effect: after 3 d of chemical treatment, the leaves of rice plant (*Oriza sativa*) were inoculated with spores of *P. oryzae*.

TABLE III. Insecticidal Activities of Daphnodorins A (1), B (2) and C (3)

Insect	Method	Mortality (%)		
		1	**2**	**3**
Spodoptera litura FABRICIUS (larvae)	Leaf dip	20	30	10
Callosobruchus chinensis L. (adult)	Leaf dip	0	30	10
Nilaparvata lugens STAL (larvae)	Stem dip	10	0	0
Tetranychus urticae KOCH (adult and egg)	Spray	30	45	11

Observation time: 5 d. Concentration: 500 ppm. Temperature: 25—27 °C.

Insecticidal Activities of Daphnodorins A (1), B (2) and C (3) on Various Noxious Insects

The insecticidal activities of compounds **1**, **2** and **3** on various noxious insects were examined. The results are summarized in Table III. Though these compounds (**1**—**3**) had no remarkable insecticidal effects, compound **2** showed weak insecticidal activities against three species of insects, *Spodoptera litura, Callosobruchus chinensis* and *Tetranychus urticae*. The insecticidal activities of **1** and **3** were weaker than those of **2**.

Discussion

It was found that three flavans, daphnodorins A (**1**), B (**2**) and C (**3**), isolated from the root and bark of *Daphne odora* THUNB., showed antifungal and insecticidal activities.

First, compounds **1**—**3** showed rather strong antifungal activities against *Pyricularia oryzae* (Table I). However, these compounds did not show antifungal activities against other plant-pathogenic fungi examined. The specific antifungal activities of these compounds (**1**—**3**) on *P. oryzae* are interesting; although various antibiotics such as kasugamycin,[3] blasticidin S,[4] antimycin[5] and polyoxins[6] have been found to inhibit the growth of *P. oryzae*, there is no report about constituents of plants which have antifungal activity against this fungus. The relatively strong antifungal activities of these compounds (**1**—**3**) are noteworthy since the source plant is used in traditional medicine as a crude drug. Compounds **1** and **3** showed residual effects of 48 and 40%, respectively, on leaves at the concentration of 500 ppm (Table II). Considering that few low-toxicity antifungal substances acting on *P. oryzae* have been discovered as yet, it would be worthwhile to carry out chemical modification studies of compounds **1**—**3** with the aim of enhancing the antifungal and residual effects. Compound **2** also showed weak insecticidal activity against three species of insects (Table III). As mentioned above, four daphnane-type diterpene esters having nematicidal and other biologi-

cal activities[2] have already been isolated from this plant. We have also found that these compounds (**1**—**3**) have weak inhibitory effects against *C. elegans* (data not shown).

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Evaluation of Indomethacin Sustained-Release Suppositories Using a Hydroxypropylmethylcellulose Acetate Succinate–Polyethylene Glycol 2000 Solid Matrix[1]

NORIAKI OHNISHI,*[a] YOSHIFUMI KIYOHARA,[a] YOSHIAKI KITA,[a] KOJI KURODA[a] and TERUYOSHI YOKOYAMA[b]

Hospital Pharmacy, Kobe University School of Medicine,[a] Kusunoki-cho, Chuo-ku, Kobe 650, Japan and Kyoto Pharmaceutical University,[b] 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan

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Indomethacin (IM) sustained-release suppositories were prepared by using a solid matrix of cellulose acetate phthalate (CAP) or hydroxypropylmethylcellulose acetate succinate (AS·MF) as a poorly soluble carrier and polyethylene glycol 2000 (PEG 2000) as a soluble carrier, and the utility of AS·MF as a poorly soluble carrier was compared with that of CAP. The release rate of IM from the AS·MF matrix suppositories, as well as that from the CAP matrix suppositories, decreased with increase of the content of AS·MF. The sustained release of IM from the AS·MF matrix suppositories was attributed to the formation of a network structure of AS·MF. Rectal administrations of the CAP or AS·MF matrix suppositories in rabbits resulted in good sustained-release characteristics. The bioavailability of the AS·MF matrix suppositories was higher than that of the CAP matrix suppositories.

Keywords——indomethacin; sustained-release suppository; solid dispersion; cellulose acetate phthalate–polyethylene glycol matrix; hydroxypropylmethylcellulose acetate succinate–polyethylene glycol matrix; X-ray diffraction; *in vitro* release rate; rectal administration; bioavailability

In the previous papers,[2,3] we reported that nifedipine suppositories prepared by using a solid matrix of cellulose acetate phthalate (CAP) as a poorly water-soluble carrier and polyethylene glycol 4000 (PEG 4000) as a water-soluble carrier showed a sustained-release characteristic and good bioavailability in rabbits and humans. Furthermore, we reported that indomethacin (IM) sustained-release suppositories prepared with a hydroxypropylmethylcellulose phthalate 200731 (HP55)–PEG 2000 solid matrix showed good bioavailability in rabbits.[4]

In this study, we examined the utility of hydroxypropylmethylcellulose acetate succinate (AS·MF), a newly developed enteric coating agent, as a poorly soluble carrier in IM sustained-release suppositories.

Experimental

Materials——IM and AS·MF were gifts from Sumitomo Pharmaceutical Co., Ltd. and Shin-etsu Chemical Co., Ltd., respectively. CAP and PEG 2000 were supplied by Wako Pure Chemical Ind., Ltd. All other chemicals were reagent-grade commercial products.

Preparation of Suppositories——Conventional Suppositories (C-0): C-0 was prepared by the fusion method using IM and PEG 2000 as a base.

Matrix Suppositories: Matrix suppositories were prepared by using CAP–PEG 2000 and AS·MF–PEG 2000 matrices as a base according to the fusion method as previously reported.[4] The matrix suppositories containing 5, 10 and 15% CAP were termed CAP-5, CAP-10 and CAP-15, and those containing 5, 10 and 15% AS·MF were termed AS·MF-5, AS·MF-10 and AS·MF-15, respectively.

TABLE I. Formulae of Suppositories[a] and Matrices

	IM (g)	CAP (g)	AS·MF (g)	PEG 2000 (g)
C-0	0.25	—	—	9.75
CAP-5	0.25	0.50	—	9.25
CAP-10	0.25	1.00	—	8.75
CAP-15	0.25	1.50	—	8.25
AS·MF-5	0.25	—	0.50	9.25
AS·MF-10	0.25	—	1.00	8.75
AS·MF-15	0.25	—	1.50	8.25

a) The weight of each suppository was 1 g, and the IM content in all suppositories was 25 mg.

The formulae of suppositories prepared in this study are listed in Table I. The content of IM in all suppositories and the suppository weight were 25 mg and 1 g, respectively. All suppositories were stored in a desiccator at room temperature, and were administered within 24 h after preparation.

X-Ray Diffractometry——The samples for determination of the crystallinity of IM in a matrix were prepared according to the same method as described in the previous paper.[4] The formulae of matrices are listed in Table I. All matrices were stored in a desiccator at room temperature. The X-ray diffraction spectra were determined with an X-ray diffractometer (Miniflex, Rigaku Denki, Ltd.; Cu-K_α radiation, 30 kV, 10 mA).

Release Test of Suppositories *in Vitro*——The release test was performed at 37 °C with a suppository release test apparatus (Toyama Ind., Ltd.) according to the same method as reported previously.[4] Five hundred milliliters of 0.1 M phosphate buffer solution (pH 7.2; μ=0.5, NaCl) was used as the test solution.

Scanning Electron Microscopy——The surface of matrix suppositories was observed with a scanning electron microscope (Nihon Denshi, JSM-T20).

Animal Experiments——White male rabbits weighing from 2.6 to 4.0 kg were fasted for 36 h prior to the experiments but were allowed free access to water. After rectal administration of a test suppository, blood samples were collected from the ear vein at regular intervals. The plasma samples were frozen and stored at −5 °C until assay.

Assay of IM in Plasma——The concentrations of IM in the plasma were determined by high-performance liquid chromatography as reported in the previous paper.[4]

Results and Discussion

Crystallinity of IM in Matrices

Figure 1 shows the X-ray diffraction spectra of IM powder, CAP-15 and AS·MF-15. There were no characteristic peaks of IM crystals (*e.g.*, 11.5, 16.6 and 21.8° (2θ)) in the spectra of CAP-15 and AS·MF-15. Two major peaks at about 19 and 23° (2θ) in these spectra were identified as being attributable to PEG 2000. These results indicate that IM is present in an amorphous state in these matrices.

The effect of the content of CAP or AS·MF on the crystallinity of IM in matrices during storage is shown in Table II. C-0 showed characteristic peaks due to IM crystals after storage for 1 month. On the other hand, CAP-5, CAP-10 and CAP-15, as well as AS·MF-5, AS·MF-10 and AS·MF-15, did not show any peak attributable to IM crystals after storage for 2 months. These results suggest that CAP and AS·MF are able to inhibit the crystallization of IM in these matrices. Sugimoto *et al.*[5] reported that the crystallization of a poorly water-soluble drug in a polymer might be related to the hygroscopicity of the polymer. Therefore, the hygroscopicity of PEG 2000 may have been decreased because of the addition of CAP or AS·MF to PEG 2000 in this study.

Release Patterns of IM from Suppositories *in Vitro*

Figure 2 shows the effect of the content of CAP or AS·MF on the release patterns of IM from suppositories. The release rate of IM from C-0 was very high and C-0 was dissolved within 20 min. However, the release rates of IM from the CAP or AS·MF matrix sup-

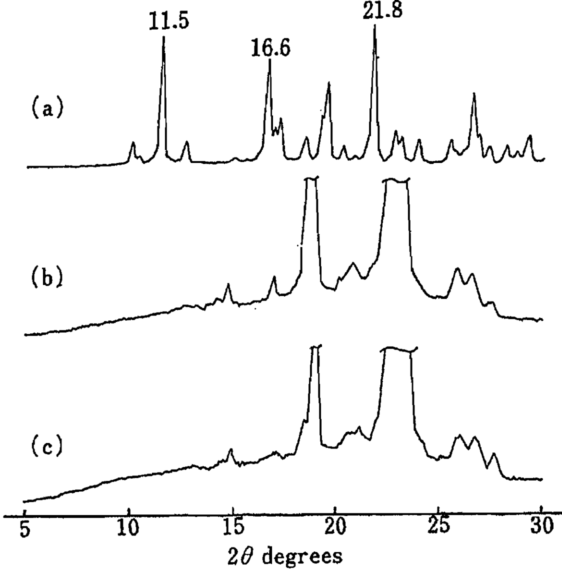

Fig. 1. X-Ray Diffraction Spectra of Matrices

(a) IM powder, (b) CAP-15, (c) AS·MF-15, IM content 2.5%, CAP and AS·MF content 15%. Range: (a) 240000 cpm; (b, c) 120000 cpm.

Table II. Crystallinity of IM in Matrices Stored in a Desiccator at Room Temperature

Matrix	Before storage	After 1 month	After 2 months
C-0	−	+	+
CAP-5	−	−	−
CAP-10	−	−	−
CAP-15	−	−	−
AS·MF-5	−	−	−
AS·MF-10	−	−	−
AS·MF-15	−	−	−

+, X-ray diffraction peaks of IM crystals appeared; −, no X-ray diffraction peaks of IM crystals appeared.

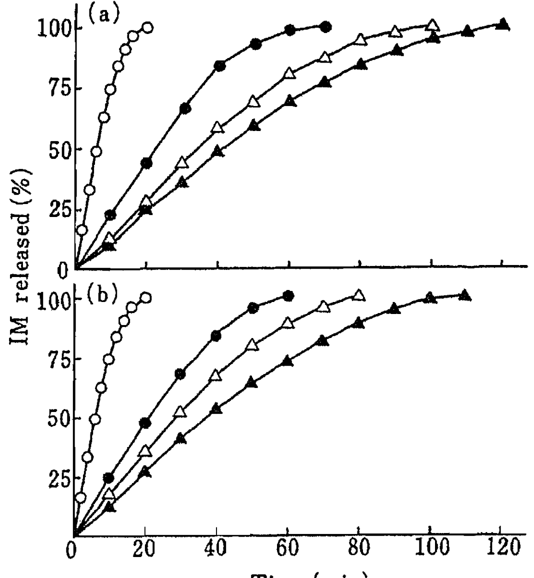

Fig. 2. Effect of the Content of CAP or AS·MF on the Release Behavior of IM from Suppositories *in Vitro*

(a) ○, C-0; ●, CAP-5; △, CAP-10; ▲, CAP-15. (b) ○, C-0; ●, AS·MF-5; △, AS·MF-10; ▲, AS·MF-15. Each point represents the mean of three experiments.

positories were low, and decreased with increase of CAP or AS·MF content. The release rate of the AS·MF matrix suppositories tended to be higher than that of the CAP matrix suppositories at the same content of CAP and AS·MF. This may be due to the difference of dissolution rates of CAP and AS·MF in the test solution. These results indicate that the content and species of the poorly soluble carrier affect the release rate of IM from the matrix suppositories.

On the other hand, the release behavior of the matrix suppositories stored for 2 months was the same as that before storage (the data were not shown). From these results and the X-ray diffraction data (Table II), it was concluded that the matrix suppositories were physically stable for at least 2 months in a desiccator at room temperature.

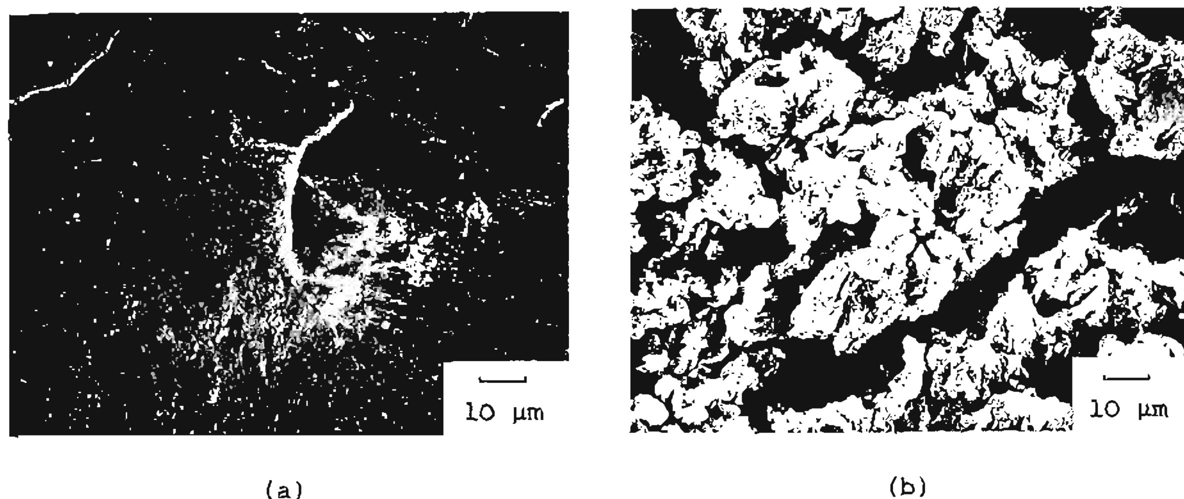

Fig. 3. Scanning Electron Micrographs of the Surface of AS·MF-15

(a) before the release test, (b) at 15 min after the start of the release test in 0.1 M phosphate buffer solution (pH 7.2) at 37 °C.

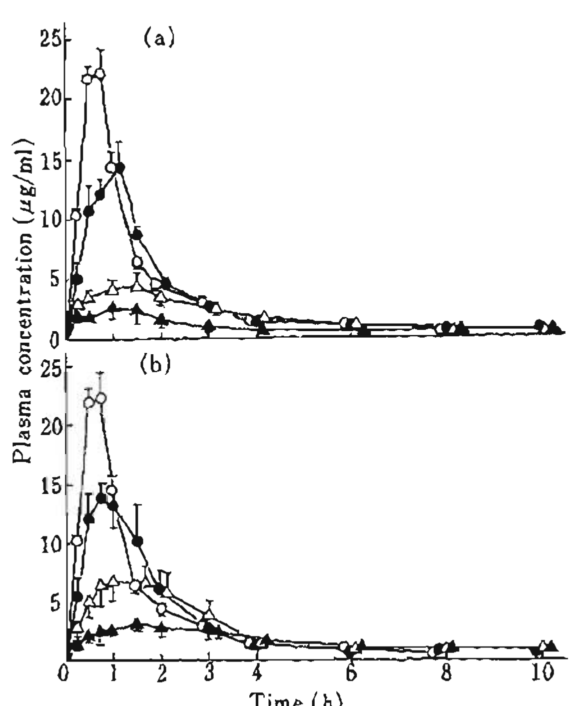

Fig. 4. Plasma Levels of IM after Rectal Administration of Suppositories in Rabbits

(a) ○, C-0; ●, CAP-5; △, CAP-10; ▲, CAP-15. (b) ○, C-0; ●, AS·MF-5; △, AS·MF-10; ▲, AS·MF-15. Each point represents the mean ± S.E. of 3—6 rabbits.

Release Mechanism of IM from Matrix Suppositories

The sustained-release mechanism of a drug from the CAP matrix suppositories has already been elucidated in the previous paper.[2] In this study, the release mechanism of IM from the AS·MF matrix suppositories was investigated by scanning electron microscopy. As shown in Fig. 3, many pores could be observed at the surface of AS·MF-15 at 15 min after the start of the release test. These results show that the sustained-release mechanism in this case is similar to that of the CAP or HP55 matrix suppositories.[2,4]

Plasma Levels of IM after Rectal Administration and Bioavailability in Rabbits

The plasma levels of IM after rectal administration of suppositories in rabbits are shown

Table III. Bioavailability Parameters[a] after Rectal Administration of Suppositories (IM: 25 mg) in Rabbits

	$n^{b)}$	Body weight (kg)	C_{max} (μg/ml)	$AUC_0^{10\,c)}$ (μg·h/ml)	*EBA* (%)
C-0	4	3.5±0.1	22.2±2.0	32.1±2.1	100.0
CAP-5	4	3.8±0.2	14.4±2.2	28.7±5.4	89.4
CAP-10	4	3.1±0.1	4.4±1.1	17.5±1.4	54.5
CAP-15	3	2.9±0.1	2.7±1.1	9.5±2.2	29.6
AS·MF-5	4	3.7±0.2	13.9±1.4	31.1±4.6	97.8
AS·MF-10	6	3.7±0.2	6.8±1.7	24.7±5.2	76.9
AS·MF-15	5	3.7±0.2	3.1±0.8	15.5±2.9	48.3

a) Each value represents the mean±S.E. *b*) The number of rabbits used. *c*) Calculated by use of the trapezoidal rule from 0 to 10 h.

in Fig. 4. The absorption of IM after administration of C-0 was very fast, and the plasma level reached a peak of 22.2 μg/ml at 45 min and then declined rapidly. CAP-5 and CAP-10 gave peaks of 14.4 μg/ml at 60 min and 4.4 μg/ml at 90 min, respectively. The administration of CAP-15 resulted in a low plasma level from 15 min to 10 h (Fig. 4a). On the other hand, AS·MF-5 showed a high peak of 13.9 μg/ml at 45 min and AS·MF-10 gave a peak of 6.8 μg/ml at 60 min. The administration of AS·MF-15 resulted in a low plasma level from 15 min to 10 h (Fig. 4b).

The area under the plasma concentration–time curve (*AUC*) and the extent of bioavailability (*EBA*) after rectal administration are listed in Table III. The *EBA* of the CAP or AS·MF matrix suppositories decreased with increase of the content of CAP or AS·MF, and also the *EBA* of the AS·MF matrix suppositories was higher than that of the CAP matrix suppositories. For instance, the *EBA* of AS·MF-15 with a good sustained-release characteristic was about 1.6 times that of CAP-15. These results suggest that AS·MF-15 is superior to CAP-15. However, the *EBA* of AS·MF-15 was only half that of C-0. It may be that the content of AS·MF is so high that the PEG-entrapped IM at the inner portion of the suppository can not be released, and the superficial erosion proceeds more slowly as described in the previous paper.[4] Therefore, it should be possible to enhance the bioavailability of AS·MF-15 by incorporating IM in the outside layer of AS·MF-15 or by miniaturizing AS·MF-15, as reported in the previous papers.[2,4]

Consequently, it appears that AS·MF is useful as a poorly soluble carrier in IM sustained-release suppositories with a combination of a poorly soluble carrier and PEG.

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Dissolution Properties of Indomethacin from Coprecipitates with Water-Insoluble Glucan

KEN MASUMOTO,*[,a] AKIYOSHI YOSHIDA,[a] MASAMI HIRAYAMA,[a] SHIN'ICHI HAYASHI,[b] YOSHIHARU MACHIDA,[c] and TSUNEJI NAGAI[c]

The Application and Development Division,[a] the Research and Development Division[b] of Rohto Pharmaceutical Co., Ltd., Tatsumi Nishi 1-8-1, Ikuno-ku, Osaka 544, Japan, and Faculty of Pharmaceutical Sciences, Hoshi University,[c] Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan

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The dissolution properties of indomethacin (IMC) from coprecipitates with water-insoluble glucan (WIG) were investigated with a view to application of WIG to pharmaceutical preparations. WIG was produced by an enzyme present in the culture fluid of *Streptococcus mutans*. Coprecipitates were prepared by evaporation of the solvent from IMC solution in which WIG was suspended. The X-ray diffraction patterns indicated that the size of crystals of IMC was decreased by the coprecipitation. The dissolution rate of IMC from the coprecipitates was significantly greater than that from the physical mixture or intact IMC powder. These results indicate that WIG can improve the dissolution properties of IMC.

Keywords——dissolution property; indomethacin; coprecipitate; water-insoluble glucan; *Streptococcus mutans*

In oral administration of poorly water-soluble drugs, the bioavailability depends mainly on the dissolution rate of the drug in the gastrointestinal fluid.[1] Therefore, if the dissolution rate of such a drug were increased, the bioavailability might be improved, and consequently side effects might be reduced since a lower dose could be employed. From such a viewpoint, many studies have been carried out on means for enhancing the solubility and dissolution rate of poorly water-soluble drugs by the utilization of coprecipitates with polymers such as polyvinylpyrrolidone.[2]

The authors have reported a method for the production of water-insoluble glucan (WIG)[3] and have demonstrated the usefulness of WIG as a vehicle for directly compressed tablets[4] and sustained release tablets.[5] In the present study, with a view to further application of WIG to pharmaceutical preparations, the effect of coprecipitation with WIG on the dissolution properties of a poorly water-soluble drug was investigated. Indomethacin, a practically water-insoluble non-steroidal antiinflammatory drug, was used as a model substance.

Experimental

Materials——WIG obtained by the method described in the previous paper[3] and indomethacin (IMC) of JP XI grade were used after being passed through a 100—200 mesh sieve.

Preparation of Physical Mixture——A physical mixture of IMC with WIG in a 1 : 3 weight ratio was prepared by simple blending in a mortar.

Preparation of Coprecipitate——IMC was dissolved and WIG was suspended in 1 : 1, 1 : 2 and 1 : 3 weight ratios in ethanol at 50 °C. The solvent was removed *in vacuo* using a rotary evaporator at 50 °C, then the residue was dried *in vacuo* at room temperature for 24 h, ground in a mortar and used after being passed through a 100—200 mesh sieve.

Powder X-Ray Diffraction Study——Powder X-ray diffractometry was carried out using a diffractometer (Geigerflex model D-2, Rigaku Denki, Tokyo, Japan) with Ni-filtered, Cu-K_α radiation.

Dissolution Rate Study——The dissolution rate study was carried out by a beaker method. Sample powder, which corresponded to 25 mg of IMC, was dispersed in 50 ml of pH 6.0, 1/15 M phosphate buffer solution at 37 °C. After the addition of the powder, vigorous agitation was applied with a magnetic stirrer. At appropriate time intervals, 1 ml of the solution was taken out with a 1 ml transfer pipette and filtered with a membrane filter (TM-2 0.45 μm, Toyo Roshi, Tokyo, Japan). The concentration of IMC in the filtrate was measured by the ultraviolet (UV) absorption method at 265 nm using a spectrophotometer (model UV-240, Shimadzu Corp., Kyoto, Japan) after dilution with 1/15 M phosphate buffer solution at pH 6.0. WIG has no UV absorption at 265 nm and did not affect the measurement of IMC.

Results and Discussion

Powder X-Ray Diffraction Study

Powder X-ray diffraction patterns of IMC powder, WIG powder, physical mixture and coprecipitates are shown in Fig. 1. Several sharp diffraction peaks attributed to IMC crystals can be seen in the case of the physical mixture. However, those peaks were almost absent in the coprecipitates of all weight ratios, suggesting a decrease in the crystallinity of IMC as a result of the coprecipitation.

Dissolution Study

Dissolution profiles of IMC from the coprecipitates are shown in Fig. 2 in comparison with those from the physical mixture or intact IMC powder. The dissolution rates of IMC from the coprecipitates were significantly greater than that from IMC alone or the physical mixture. The dissolution rate of IMC from the coprecipitates increased as the ratio of WIG to

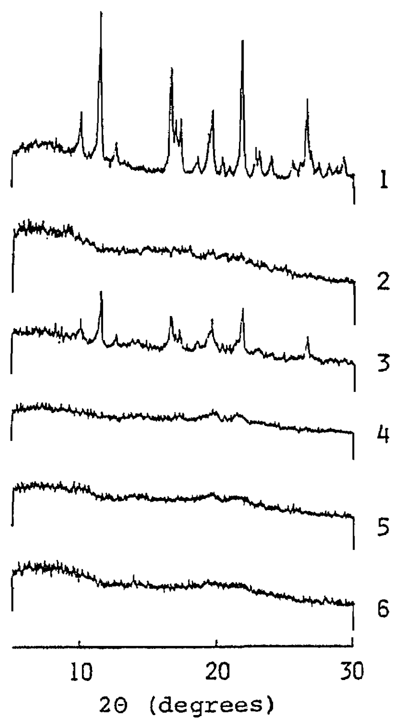

Fig. 1. Powder X-Ray Diffraction Patterns of IMC–WIG Coprecipitates in Different Ratios

1, IMC alone; 2, WIG alone; 3, physical mixture (1 : 3); 4, coprecipitate (1 : 1); 5, coprecipitate (1 : 2); 6, coprecipitate (1 : 3).

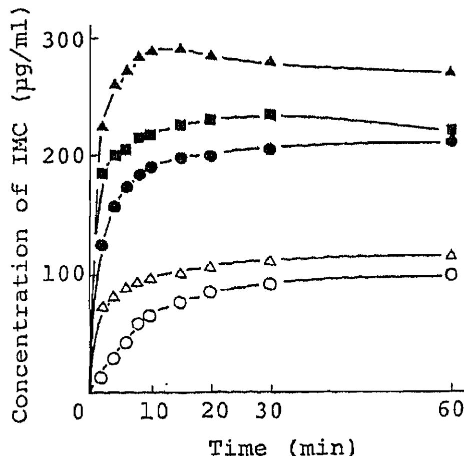

Fig. 2. Dissolution Profiles of IMC from Coprecipitates with WIG

●, IMC : WIG = 1 : 1 coprecipitate; ■, 1 : 2 coprecipitate; ▲, 1 : 3 coprecipitate; △, 1 : 3 physical mixture; ○, IMC alone. Each point represents the mean of three determinations.

IMC was increased. When the ratio of IMC to WIG was 1:3 or 1:2, the concentration of IMC reached a peak after 15 or 30 min, respectively, then decreased gradually. Since the solubility of IMC in this medium at 37 °C was 160.0 μg/ml, supersaturation occurred in the initial stage of dissolution of the coprecipitate. IMC in the physical mixture dissolved more rapidly than IMC alone in the initial stage. The difference of dissolution of IMC between the physical mixture and IMC alone may be explained by the additive dispersion effect as demonstrated by Shah *et al.*[6]

Thus, coprecipitation of IMC with WIG reduced the crystallinity of IMC, and the dissolution rate of IMC was enhanced. Therefore, the method of coprecipitation of poorly water-soluble drugs with WIG might be useful to improve the bioavailability and to reduce the side effects of such drugs.

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Classification and Determination of N-Nitroso Compounds Based on the Differences in Reactivity to Denitrosating Reagents

TAKAFUMI OHTA,* KIMIKO KOJIMA and SHOJI TAKITANI

Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162, Japan

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The denitrosation of eleven N-nitroso compounds was followed by using a modification of the method of Eisenbrand and Preussmann. N-Nitroso compounds (except nitrosamides) whose precursor amines had pK_a values smaller than about 10.7 were found to be denitrosated instantaneously on addition of hydrogen bromide/acetic acid. These easily denitrosatable compounds were determined selectively with hydrogen chloride/acetic acid. The order of increasing difficulty in denitrosation of N-nitroso compounds as judged from the reactivity with the above reagents coincided well with that of pK_a values of the precursor amines.

Keywords——spectrophotometric determination; classification; N-nitroso compound; denitrosation reagent; nitrosodimethylamine; nitrosoproline

Determination of N-nitroso compounds is of considerable concern since they form a class of potent carcinogens. Eisenbrand and Preussmann[1] developed a spectrophotometric method for determination of nitrosodialkylamines based on the denitrosation reaction with hydrogen bromide/acetic acid followed by reaction of the liberated nitrite with Griess reagent. As the specificity of this denitrosation reaction to N-nitroso compounds is well established,[2,3] it has been used to analyze total N-nitroso compounds in foods and nitrite-treated drugs.[4-6] However, the denitrosation process has not been well characterized except for nitrosodimethylamine.[1] During the course of determination of *N*-nitrosoproline by the method of Eisenbrand and Preussmann,[1] we found that this compound was denitrosated instantaneously, in contrast to nitrosodimethylamine, on addition of hydrogen bromide/acetic acid, indicating that certain N-nitroso compounds might be determined selectively depending on the denitrosation conditions employed. The development of such a selective method would be useful to characterize unknown N-nitroso compounds, because in foods, the cumulative amounts of known N-nitroso compounds determined separately account for only a part of total N-nitroso compounds.[7]

In the present study, the denitrosation of various N-nitroso compounds with different reagents was followed by using a modification of the procedure of Eisenbrand and Preussmann,[1] and N-nitroso compounds whose precursor amines had smaller pK_a values were found to be denitrosated more easily. Such easily denitrosatable N-nitroso compounds could be determined selectively by the use of hydrogen chloride/acetic acid.

Materials and Methods

Chemicals——Nitrosodimethylamine (NDMA) and nitrosodiisobutylamine (NDBA) were purchased from Wako Pure Chemical Co., *N*-nitroso-*N*-methylaniline (NMA), *N*-nitrosomorpholine (NMP) and *N*-nitrosodiphenylamine (NDPA) were from Tokyo Kasei Kogyo Co., *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and *N*-nitroso-*N*-methylurea (NMU) were from Nakarai Chemicals Co., and *N*-nitrosopyrrolidine (NPYR) and *N*-nitroso-*N*-methylurethane (NMUT) were from Aldrich Chemical Co. Nitrosoproline (NPRO) and nitrososarcosine

(NSAR) were synthesized according to the methods of Lijinsky *et al.*[8] and Yamamoto *et al.*,[9] respectively.

The denitrosation reagents were prepared as follows. Hydrogen bromide/acetic acid (1.88% HBr/HOAc); mix 2.7 ml of 47% HBr, 12 ml of acetic anhydride and 80 ml of HOAc. Hydrogen chloride/acetic acid (1.88% HCl/HOAc); mix 4.5 ml of 35% HCl, 18.5 ml of acetic anhydride and 71 ml of HOAc. Griess reagent (0.5% sulfanilamide–0.05% *N*-1-naphthylethylenediamine dihydrochloride in 30% HOAc) was prepared freshly before use. Sulfanilamide was used instead of sulfanilic acid because of its higher solubility in 30% HOAc.

Determination of Total N-Nitroso Compounds——The method of Eisenbrand and Preussmann[1] was modified as follows. To 0.5 ml of ethyl acetate sample solution was added 2 ml of HBr/HOAc, and the mixture was allowed to stand for 10 min at room temperature. Griess reagent (4 ml) was then added, and the absorbance at 550 nm was recorded after 15 min.

Determination of Easily Denitrosatable N-Nitroso Compounds——HCl/HOAc was used instead of HBr/HOAc. Other procedures were the same as in the case of total N-nitroso compounds. Of these easily denitrosatable compounds, extremely easily denitrosatable ones such as NDPA and NMA could be estimated roughly by using cooled sample solution (0 °C) and HCl/HOAc (not below 16 °C), and adding Griess reagent immediately after addition of HCl/HOAc.

Results and Discussion

Ethyl acetate, instead of acetic acid, was used as a sample solvent in the present study. About 10% decrease in denitrosation efficiency was observed when ethyl acetate was used for determination of NDMA by the recommended procedure with HBr/HOAc. Ethyl acetate was nevertheless employed, because acetic acid seemed impractical for extraction and concentration of N-nitroso compounds from various samples.

Figure 1 shows the denitrosation process of NDMA and NPRO with HBr/HOAc. NDMA was denitrosated gradually, while NPRO was denitrosated instantaneously on addition of HBr/HOAc. We therefore examined nine other N-nitroso compounds. NMA, NMP, NSAR, NDPA and MNNG showed NPRO-type processes, while NDBA, NMU, NMUT and NPYR showed NDMA-type processes. The absorbance for these NPRO-type compounds, except NMA and NDPA, was negligible when the order of addition of HBr/HOAc and Griess reagent was reversed. The absorbance for NMA and NDPA in such a case amounted to about 10% and 100% of that obtained by the recommended procedure, respectively, indicating that NDPA is extremely susceptible to denitrosation. NDPA gave only 5% of the absorbance obtained by the recommended procedure when treated with HOAc alone, instead of HBr/HOAc, for a very short period.

The denitrosation yields of all the compounds examined are summarized in Table I. All the N-nitroso compounds were denitrosated in over 80% yield when treated by the recommended procedure with HBr/HOAc.

The above results indicate that N-nitroso compounds can be classified into at least two groups based on the ease of their denitrosation, and that the easily denitrosatable compounds may be determined separately by adding Griess reagent immediately after addition of HBr/HOAc. However, immediate and precise addition of Griess reagent was difficult to control,

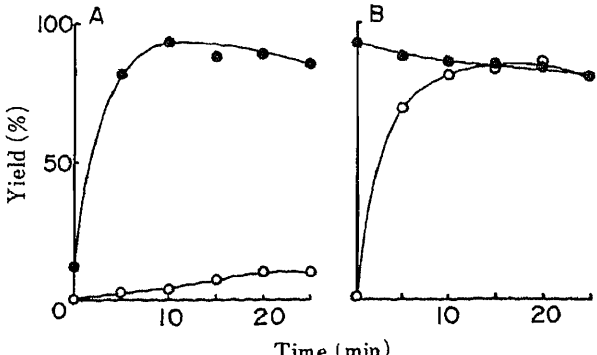

Fig. 1. Time Courses of Denitrosation of NDMA (A) and NPRO (B) with HBr/HOAc and HCl/HOAc

NDMA and NPRO (135 nmol each) were each treated with HBr/HOAc (●) and HCl/HOAc (○) for various times according to the recommended procedure. Each value in the figure represents the mean of duplicate runs. Yields were calculated with each reagent on the basis of the corresponding amount of nitrite.

TABLE I. Denitrosation Efficiency of N-Nitroso Compounds with HBr/HOAc and HCl/HOAc

Compound	Yield (%)[a]			
	HBr/HOAc		HCl/HOAc	
	Reaction time (min)			
	0[b]	10	0[b]	10
NDPA	93	83	93	82
NMA	102	97	74	87
NMP	98	87	1	79
NSAR	104	94	0	85
NPRO	92	86	1	80
NDBA	40	96	1	6
NDMA	12	93	0	4
NPYR	4	93	0	1
NMU	7	79	3	77
NMUT	22	84	10	85
MNNG	97	87	19	84

a) Yields were calculated on the basis of the corresponding amount of nitrite. *b*) Griess reagent was added immediately after addition of denitrosating reagent. Each value represents the mean of duplicate runs.

and the absorbance obtained for NDMA-type compounds by using this technique tended to increase if a slight delay occurred in addition of the reagent. Further, NDBA, though classified as being of NDMA type, was denitrosated in 40% yield when treated by this technique (Table I). Cooling the sample solution and HBr/HOAc reagent before mixing selectively decreased the absorbance of NDMA-type compounds at "zero time": NDBA was denitrosated in only 6% yield at "zero time". However, we did not employ this technique, because it was tedious to cool the HBr/HOAc reagent without freezing.

Figure 1 also shows the denitrosation processes of NDMA and NPRO with HCl/HOAc. NPRO was denitrosated gradually, while NDMA was only slightly denitrosated even on prolonged treatment: NDMA was denitrosated in only 4% yield when treated by the recommended procedure with HCl/HOAc (Table I). NMP, NSAR, MNNG, NMU and NMUT showed NPRO-type processes with this reagent. NDBA and NPYR showed similar patterns to NDMA. NMA and NDPA were denitrosated instantaneously. NDPA was denitrosated to almost the same extent even when the order of addition of HCl/HOAc and Griess reagent was reversed. The denitrosation yields are summarized in Table I. All the N-nitroso compounds, except NDBA, NDMA and NPYR, were denitrosated in 77—87% yield when treated by the recommended procedure with HCl/HOAc.

The above results indicate that easily denitrosatable N-nitroso compounds can be determined selectively with HCl/HOAc reagent. The experiments with model sample mixtures containing various amounts of NDMA and NPRO (0—0.27 μmol each) showed that NPRO could be determined selectively with HCl/HOAc in the presence of NDMA (103—115% recovery), and that NDMA could be determined by subtracting the NPRO from total N-nitroso compounds measured with HBr/HOAc (80—106%).

N-Nitroso compounds that were denitrosated gradually, such as NDMA with HBr/HOAc or NPRO with HCl/HOAc, gave curved calibration lines at concentrations above 0.6 μmol/ml ($r=0.9990$ for NDMA, below 0.54 μmol/ml; coefficient of variation at 0.135 μmol/ml, 3.2%, $n=10$). On the other hand, N-nitroso compounds that were de-

nitrosated instantaneously, such as NPRO with HBr/HOAc or NDPA with HCl/HOAc, gave linear calibration lines up to at least 0.8 μmol/ml (r=0.9997 for NPRO; coefficient of variation at 0.135 μmol/ml, 2.0%, n=10). Therefore, care should be taken when the present methods are applied to analysis of unknown mixtures: simultaneous runs with a diluted sample solution are preferable.

The specificity of the recommended methods with HBr/HOAc and HCl/HOAc was examined using 0.27 μmol/ml solutions of nitromethane, acetoxime, nitrosobenzene, nitrobenzene, butyl nitrite and *N*-nitrodimethylamine. Only butyl nitrite gave a positive result (efficiency, 97% with HBr/HOAc; 90% with HCl/HOAc). Such interference by alkyl nitrites has been reported by previous workers.[2,3]

NMA and NDPA may be determined separately from other easily denitrosatable compounds by choosing an appropriate reagent instead of HCl/HOAc. Hydrogen fluoride, trifluoroacetic acid or methanesulfonic acid in HOAc was examined: hydrogen iodide in HOAc was not examined because of its susceptibility to oxidation. Unsatisfactory results, however, were obtained with these reagents. Cooling the sample solution and HCl/HOAc, and adding Griess reagent immediately after addition of HCl/HOAc, though tedious and liable to significant experimental error, seems to be the only technique currently available to estimate NMA and NDPA. The yield in the case of NDPA or NMA was hardly affected by the cooling, while that of MNNG decreased slightly (13%).

The order of ease of denitrosation of the N-nitroso compounds examined is shown in Table II, together with the pK_a values of the precursor amines. The order of increasing difficulty in denitrosation coincided well with that of pK_a values of precursor amines except for MNNG, NMU and NMUT. The nitroso group of N-nitroso compounds has been considered to be removed as nitrosyl bromide with HBr.[1] The above correlation between pK_a value and ease of denitrosation, therefore, seems reasonable. It is interesting that MNNG, NMU and NMUT are exceptional, since the precursor amides of these nitrosamides are nitrosated with different kinetics from those that common secondary amines obey.[10] These nitrosamides may be denitrosated in a different manner.

Table II. Order of Ease of Denitrosation and pK_a

Compounds	Order[a]	pK_a[b]	Compounds	Order[a]	pK_a[b]
NDPA	1	0.79	NDBA	7	10.91
NMA	2	4.85	NDMA	8	10.73
MNNG	3	13.40[c]	NPYR	9	11.27
NMP	4 or 5	8.33	NMU	—	0.70
NSAR	4 or 5	10.12	NMUT	—	—
NPRO	6	10.64			

a) The order was determined on the basis of the reactivity of each compound with two denitrosating reagent (Table I) and the time needed for maximal yield. *b*) pK_a of precursor amine (most pK_a values are taken from Weast and Astle[11]). *c*) pK_a of methylguanidine.

In conclusion, the recommended method with HBr/HOAc could determine total N-nitroso compounds, and that with HCl/HOAc could determine easily denitrosatable NPRO-type compounds whose precursor amines had pK_a values smaller than about 10.7. Of these easily denitrosatable compounds, those whose precursor amines had pK_a values smaller than about 4.8 could be estimated roughly by adding Griess reagent immediately after addition of HCl/HOAc.

A chemiluminescence determination of total nitrosamides using concentrated hydrochloric acid as a denitrosation reagent has been developed recently.[12] All the nitrosamides

examined, however, give twice the response obtained for the corresponding amount of nitrite.[12] On the other hand, in the recommended procedure with HCl/HOAc, NDPA, NMA, NMP, NSAR and NPRO besides nitrosamides were detected, and gave absorbance similar to that obtained with a corresponding amount of nitrite (77—87%) (Table I). These discrepancies may be attributable to the differences in composition of the denitrosation reagent and in the method of detection.

The detection limit of the present method in terms of nitroso group concentration in the sample solution was about 1.5 nmol/ml. This sensitivity is insufficient for determination of general levels of N-nitroso compounds in various foods and environmental samples,[13] but seems to be adequate for determination of those formed by treating drugs[6] and foods with nitrite. The techniques presented here, therefore, should be useful to characterize unknown N-nitroso compounds in such samples.

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Communications to the Editor

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AN ANTIFEEDANT FOR THE YELLOW BUTTERFLY LARVAE IN *CAMELLIA JAPONICA*: A REVISED STRUCTURE OF CAMELLIDIN II

Atsushi Numata,* Akihiko Kitajima, Takako Katsuno, Kyoko Yamamoto, Natsu Nagahama, Chika Takahashi, Ryoko Fujiki and Michiko Nabae
Osaka University of Pharmaceutical Sciences, 2-10-65 Kawai, Matsubara, Osaka 580, Japan

Camellidin II was isolated as an antifeedant for the larvae of the yellow butterfly, *Eurema hecabe mandarina* de l'Orza, from the leaves of *Camellia japonica* L. besides a non-tested camellidin III, identified as its methyl ester, and three inactive known compounds, quinic acid, 3ß,20-dihydroxylupane, and 3,3',4-tri-*O*-methylellagic acid. Spectroscopic and chemical evidence indicates that the structure of camellidin II, reported as an antifungal saponin, is better represented as 3ß,18ß-dihydroxy-28-norolean-12-en-16-one 3-*O*-ß-*D*-glucopyranosyl-(1→2)-ß-*D*-galactopyranosyl-(1→3)-[ß-*D*-galactopyranosyl-(1→2)]-ß-*D*-glucuronopyranoside.

KEYWORDS——camellidin II; *Eurema hecabe mandarina*; *Camellia japonica*; antifeedant; saponin; multiple-relayed COSY; HETCOR; camellidin III

Our continuing search for insect antifeedants from plants [1] has led us to examine the active constituent from the leaves of *Camellia japonica* L. (Theaceae), which are inherently less severely damaged by a phytophagous insect than other plants in the field. The leaves were successively extracted by ether, methanol, hot methanol and hot water. The water extract was purified by a combination of Sephadex LH-20, XAD-II and silica gel column chromatographies and droplet countercurrent chromatography to afford compound Ia and quinic acid. The methanol extract was fractionated into chloroform soluble and water soluble fractions. The former was purified by Sephadex LH-20 and silica gel column chromatographies to yield 3ß,20-dihydroxylupane and 3,3',4-tri-*O*-methylellagic acid. The water soluble fraction was separated by XAD-II and silica gel column chromatographies and HPLC to afford compound II. Of these compounds Ia revealed antifeeding activity against the 5th instar larvae of the yellow butterfly, *Eurema hecabe mandarina* de l'Orza [feeding ratio 46.8 % (concentration 0.25 %)].

In the course of structure determination of the antifeeding active substance Ia,[2] it was found to be a mixture of sodium and potassium salts of camellidin II, which Nishino and co-workers isolated as one of antifungal saponins from the same plant.[3] Recently, the structure of camellidin II was determined as Ib by them.[4] However, our studies suggest that the structure is better represented as Ic.

In the IR spectrum, compound Ia, mp 270-275°C (dec.) (EtOH-H_2O), $[\alpha]_D^{20}$ -4.0° (c=0.86, H_2O), $C_{53}H_{83}O_{24}Na$ [FAB-MS m/z: 1127 $(M+H)^+$] or $C_{53}H_{83}O_{24}K$ [m/z: 1143

$(M+H)^+$], showed bands at 1610 and 1390 cm^{-1}, characteristic of an ionized carboxyl group, besides those at 3380 (OH) and 1705 (CO) cm^{-1}. Treatment of this compound with HCl gave camellidin II (Ic), mp 215-218°C (dec.), identical with an authentic sample, in which the COO^- absorption vanished in the IR spectrum. Acid hydrolysis of Ia afforded glucose, galactose and glucuronic acid as well as camellenodiol (III),[5] mp 213-216°C , as an aglycone.[2] For the NMR spectral analysis Ia was derivatised due to broad signals of the ^1H- and ^{13}C-NMR spectra. Acetylation of Ia gave two acetates IV and V, which were separately esterified with diazomethane to yield monomethyl esters VI and VII, respectively. Esters VI and VII contain respectively twelve and thirteen acetyl groups, the latter including C-18-OAc of the aglycone, as deduced from the NMR and mass spectra. This indicates that the sugar moiety in Ia consists of one glucuronic acid and three hexoses.

The sugar linkages of Ia were determined from the NMR data of the major ester (VI) and the chemical reaction of the acetate (IV) described as follows. The signals of four anomeric protons (A-, B-, C- and D-1), two 4-protons (B- and D-4) of ß-galactose and 5-H (C-5) of ß-glucose were easily assigned by their characteristic splitting patterns, chemical shifts and ^1H-^{13}C heteronuclear shift-correlated 2D-NMR (HETCOR) spectrum. The recently developed "multiple-relayed homonuclear shift-correlated 2D (COSY) spectrum"[6,7] showed the protons correlated with the anomeric protons (A-, B-, C- and D-1) as follows: Proton A-2 was observed in the COSY, A-2 and A-3 in the single-relayed COSY, and A-2, A-3 and A-4 in the double-relayed COSY (Fig. 1-i). Protons B-2~4, C-2~4 and D-2~4 were detected in the multiple-relayed COSY (Fig. 1-ii, iii) in the same manner. Assignments of the overlapping signals in the multiple-relayed COSY, and 5- and 6-protons were made by ^1H-^1H homonuclear decoupling (Table I). Appearance of A-5 as a doublet signal shows the sugar A to be glucuronic acid. Since two signals (B- and D-4) and one signal (C-5) correspond respectively to the protons of galactose and glucose as described above, the two sugars (B and D) and the last one (C) are galactose and glucose, respectively. Assignments of the ^{13}C-NMR signals in the sugar moieties were based on a correlation with the fully assigned proton signals

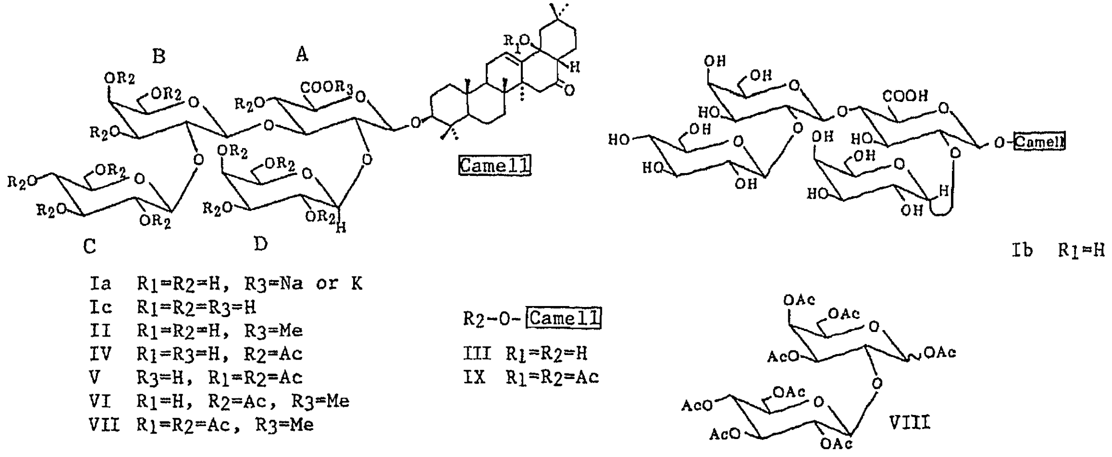

Chart 1

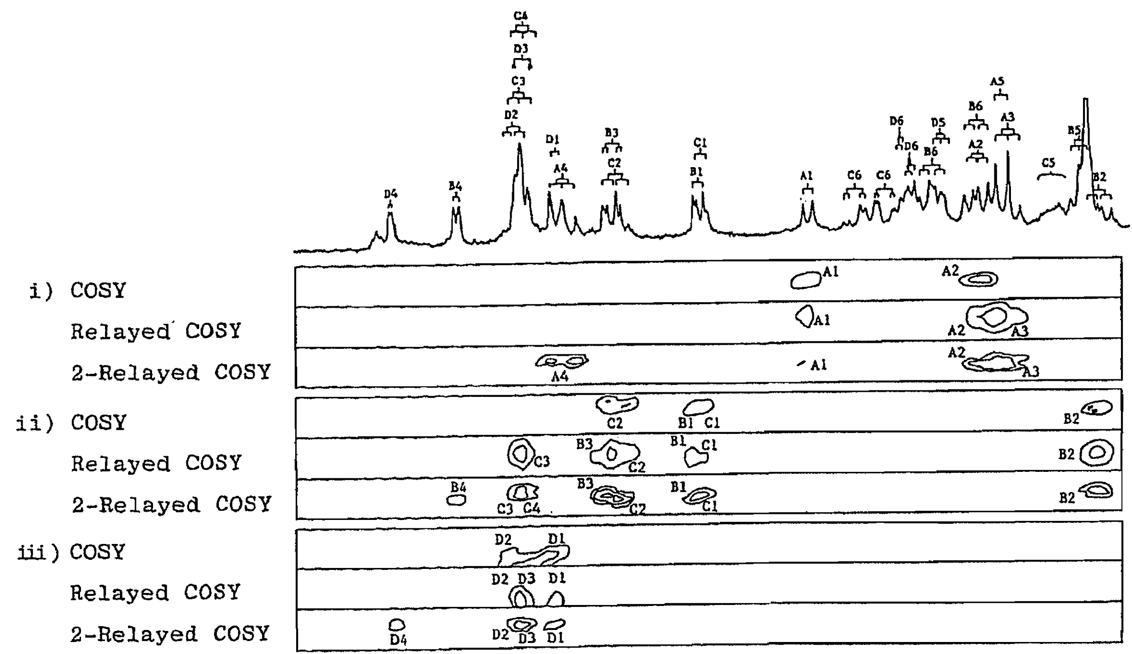

Fig. 1. Cross Sections of Multiple-Relayed COSY Spectrum of the Sugar Moiety in VI

Table I. ^{1}H and ^{13}C Chemical Shift (δ ppm) in the Sugar Units of VI in $CDCl_3$ [a)]

	Position	Proton	J_{H-H}(Hz)	Carbon	J_{C-H}(Hz)
Glucuronic acid moiety (A)	1	4.45 d	7.4	103.77 (+2.10) [b)]	157
	2	4.04 dd	8.7, 7.4	77.22 (+6.07)	
	3	3.94 t	8.7	78.71 (+6.61)	
	4	5.05 dd	9.4, 8.7	69.88 (+0.39)	
	5	3.96 d	9.4	72.55 (-0.03)	
Galactose moiety (B)	1	4.72 d	7.5	101.19 (-0.90)	163
	2	3.71 dd	10.0, 7.5	76.80 (+7.99)	
	3	4.93 dd	10.0, 3.7	72.33 (+1.35)	
	4	5.31 br d	3.7	67.10 (-0.02)	
	5	3.78 br dd	7.4, 6.6	70.39 (-0.25)	
	6	4.03 dd	10.9, 7.4	61.01 (-0.28)	
	6	4.13 dd	10.9, 6.6		
Galactose moiety (D)	1	5.07 d	6.0	99.19 (-2.90)	165
	2	5.18 dd	9.6, 6.0	70.56 (+1.75)	
	3	5.16 dd	9.6, 1.8	70.78 (-0.20)	
	4	5.48 br d	1.8	67.61 (+0.49)	
	5	4.11 br t	6.6	70.92 (+0,28)	
	6	4.20 dd	6.6, 3.6	61.06 (-0.23)	
	6	4.21 dd	6.6, 3.6		
Glucose moiety (C)	1	4.71 d	7.8	99.73 (-1.90)	164
	2	4.93 dd	9.4, 7.8	71.32 (+0.06)	
	3	5.17 t	9.4	73.03 (+0.14)	
	4	5.15 t	9.4	68.37 (-0.08)	
	5	3.84 m		71.82 (-0.02)	
	6	4.25 dd	12.5, 2.4	61.76 (-0.17)	
	6	4.33 dd	12.5, 3.8		

a) Measured on a Varian XL-300 [300 MHz (^{1}H), 75.4 MHz (^{13}C)], relative to TMS.
b) Values in parentheses are glycosidation shifts which were calculated on the basis of the ^{13}C chemical shifts of methyl tetraacetylglycoside or methyl triacetylglucuronide methyl ester.

in the HETCOR spectrum (Table I). The occurrence of glycosidation shifts[8] indicates that the glycosidic linkages exist at the 2- and 3-positions of glucuronic acid (A) and at the 2-position of galactose (B).

Acetate IV was subjected to acetic anhydride-degradation, effecting selective cleavage at the glucuronide moiety[9] to afford two anomers of 1,2,3,4,6-penta-*O*-acetyl-*D*-galactose and two anomers of the octaacetate of 2-*O*-β-*D*-glucosyl-*D*-galactoside (VIII) besides diacetylcamellenodiol (IX). Irradiation of the anomeric proton signals D- and A-1 in ester VI enhanced the respective signals of A-2 and 3-H of the aglycone in NOE difference. Alternatively, irradiation of the signals (B- and C-1) at the same time enhanced both of the signals of A-3 and B-2. In addition, the four sugar moieties all have the β-configuration as deduced from the J_{H-H} and J_{C-H} values of the anomeric protons and carbons of VI (Table I).

Based on this evidence, the structure of the ester (VI) was elucidated as VI and hence camellidin II should be represented as Ic.

Compound II, mp 260-262 °C (dec.)(EtOAc-MeOH), $[\alpha]_D^{20}$ -10.2° (c=0.24, MeOH), $C_{54}H_{86}O_{24}$ [m/z: 1141 $(M+Na)^+$], designated as camellidin III, has a methyl ester (δ 3.8 ppm) as deduced from the ^{1}H-NMR spectrum, and it formed VI and VII on acetylation, showing it to be a methyl ester of camellidin II (Ic).

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A FACILE SYNTHESIS OF HALO-1,2,3-TRIAZINES

Terumitsu Kaihoh, Takashi Itoh, Akio Ohsawa,* Mamiko Okada, Chikako Kawabata, and Hiroshi Igeta
School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Halo-1,2,3-triazines (1 and 3) were synthesized by treating 1-aminopyrazoles (2) with chlorine and bromine, without isolating intermediary triazines or halogenated 1-aminopyrazoles.

KEYWORDS——halo-1,2,3-triazine; 1-aminopyrazole; 1,2,3- triazine; halogenation; oxidation

Until recently there have been few papers pertaining to the chemical and physical properties of monocyclic 1,2,3-triazines.[1] We have been studying their syntheses and reactivities and reported that the reaction of 1,2,3-triazines with halogens afforded 5-halotriazines (1).[2] However, this reaction was limited to the preparation of 5-halo-4,6-dimethyltriazines (1d and d') in the sense of product yields. On the other hand, triazines were synthesized by oxidizing 1-aminopyrazoles (2) with metallic oxidizing reagents.[3] During our studies of the oxidation of 2, we found the reaction of 2 with halogens gave halotriazines (1 and 3).[4] The reaction offered a useful method for synthesizing the halotriazines as described below.

1-Amino-3,5-dimethylpyrazole (2d) reacted with an equimolar bromine to give 2% 5-bromo-4,6-dimethyl-1,2,3-triazine (1d)[2] and 75% 1-amino-4-bromo-3,5-dimethylpyrazole. With chlorine, 19% 5-chloro-4,6-dimethyl-1,2,3-triazine (1d')[2] was afforded, together with 2d in 40% as an HCl salt. Therefore, it was expected that the use of two equivalents of halogen would increased the yields of 5-halo-1,2,3-triazines (1). Compounds 2d-f reacted with two molar equivalents of halogens in the presence of triethylamine, which was added to neutralize the hydrogen halide generating through the reaction (method A). The reaction mixture was worked up to afford 4,6-disubstituted 5-halo-1,2,3-triazines (1d, d', e, e', and f'). In the bromination of 2a-c, the yields of 5-bromo- and 4,5-dibromotriazines (1a-c and 3a-c) were low by the above method A, but they were improved by the stepwise addition of bromine (method B). In this method, the reagents were added to a solution of 2a-c in the following order: 1)bromine (1 mol eq), 2)triethylamine (1 mol eq), and 3)bromine (1 mol eq). The reaction of 2a-c with chlorine was unsuccessful and gave complicated mixtures by both methods A and B.

Chart

Data for the 5-halo-1,2,3-triazines (1) and 4,5-dibromo-1,2,3-triazines (3) are shown in the table. The spectral data[6] supported their structures.

Table

Entry	Method	Product	R^1	R^2	Yield(%)	mp(°C)(solvent)
1	B	1a	H	H	16	125-126(dec.) (hexane)
		3a	H	Br	20	138-139(dec.) (Et_2O-hexane)
2	B	1b	Me	H	43	73- 74 (hexane)
		3b	Me	Br	8	130-132(dec.) (Et_2O-hexane)
3	B	1c	Ph	H	8	125-126(dec.) (Et_2O-hexane)
		3c	Ph	Br	10	110-112(dec.) (Et_2O-hexane)
4	A	1d	Me	Me	62	104-105 (hexane)
5	A	1d'	Me	Me	52	89- 90 (hexane)
6	A	1e	Ph	Me	54	138-140 (hexane)
7	A	1e'	Ph	Me	43	112-113 (hexane)
8	A	1f'	Ph	Ph	12	159-160 (Et_2O-hexane)

Regarding the synthesis of halotriazines, Gompper et al.[7] reported that the reaction of tetrahalocyclopropenes with trimethylsilylazide gave 4,5,6-trihalo-1,2,3-triazines. However, only 4,5,6-trichloro- and 4,5,6-tribromotriazines can be obtained by their method. Although 5-bromotriazines (1, X=Br) were also obtained by oxidizing 1-amino-4-bromopyrazoles with metallic oxidizing reagents,[2] the present method is better because it gives various halo-1,2,3-triazines (1 and 3) from available 1-aminopyrazoles (2) in a one-pot reaction without isolating the intermediary triazines or halogenated 1-aminopyrazoles.

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4) It appears that the reaction proceeds via two steps, i.e., halogenation and oxidation. According to their sequence, there are two possible paths. The more likely one would proceed through the halogenation followed by oxidation. We will report the details of this reaction including the mechanism in the near future.
5) Approximately 1:1 mixture of 3-methyl and 5-methyl derivatives.
6) 1a; ^{1}H-NMR ($CDCl_3$) δ: 9.22(s). EI-MS m/z (M^+): 159. 3a; ^{1}H-NMR ($CDCl_3$) δ: 9.11(s). EI-MS m/z (M^+): 237. 1b; ^{1}H-NMR ($CDCl_3$) δ: 2.82(3H,s), 9.07 (1H,s). EI-MS m/z (M^+): 173. 3b; ^{1}H-NMR ($CDCl_3$) δ: 2.87(s). EI-MS m/z (M^+): 251. 1c; ^{1}H-NMR ($CDCl_3$) δ: 7.54-7.61(3H,m), 7.91-8.01(2H,m), 9.23 (1H,s). CI-MS m/z (M^++1): 236. 3c; ^{1}H-NMR ($CDCl_3$) δ: 7.54-7.61(3H,m), 7.82-7.92(2H,m). CI-MS m/z (M^++1): 314. 1e; ^{1}H-NMR ($CDCl_3$) δ: 2.89(3H,s), 7.52-7.58(3H,m), 7.80-7.90(2H,m). EI-MS m/z (M^+) 249. 1e'; ^{1}H-NMR ($CDCl_3$) δ: 2.87(3H,s), 7.53-7.63(3H,m), 7.88-7.98(2H,m). CI-MS m/z (M^++1): 206. 1f'; ^{1}H-NMR ($CDCl_3$) δ: 7.55-7.62(6H,m), 7.92-8.02(4H,m). CI-MS m/z (M^++1): 238. The spectra of compounds 1d and 1d' are described in ref. 2. Satisfactory elemental analyses were obtained for all compounds.
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Communications to the Editor

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IDENTIFICATION OF 7β-HYDROXY METABOLITES OF DEHYDROCHOLATE IN MAN AND RAT

Yasuhiro Miyazaki,[*,a] Hitoshi Ichimiya,[a] Hiroshi Miyazaki,[b] Fumio Nakayama,[a] and Mitsuru Nakagaki [a]
Department of Surgery I, Kyushu University Faculty of Medicine,[a] Fukuoka 812 and Nippon Kayaku Co. Ltd.,[b] Chiyoda-ku, Tokyo 100, Japan

Dehydrocholic acid was administered intravenously to a patient and to rats, and its metabolites excreted into bile were studied by gas chromatography-mass spectrometry as ethyl ester-dimethylethylsilyl ether-methyloxime derivatives. Two 7β-hydroxy metabolites were identified: 3α,7β-dihydroxy-12-oxo-5β-cholanoic acid in both human and rat bile, and 3α,7β,12α-trihydroxy-5β-cholanoic acid in rat bile. This indicates that human and rat livers are able to reduce the 7-oxo group in dehydrocholic acid to both 7α- and 7β-hydroxy forms.

KEYWORDS —— bile acid; dehydrocholic acid; hepatic biotransformation; gas chromatography-mass spectrometry; 3α,7β-dihydroxy-12-oxo-5β-cholanoic acid

Dehydrocholic acid (DHCA), a synthetic 3,7,12-trioxo-5β-cholanoic acid, is used clinically as a choleretic and to investigate the mechanisms of bile formation. In previous studies, several investigators revealed that DHCA, when administered to man[1] and experimental animals,[2] underwent hepatic biotransformation before biliary excretion. They also demonstrated that the configuration of the hydroxy groups in the metabolites was limited to the α-configuration. Anwer and Hegner,[3] however, suggested that the keto group at C-7 in DHCA may be reduced mostly to 7β-hydroxy metabolites in rats rather than to the 7α-hydroxy forms found in man, based on the enzymatic analysis of bile acids using 3α- and 7α-hydroxysteroid dehydrogenases. Thorough characterization was still lacking and a detailed study was needed of the structure of DHCA metabolites especially at C-7. Therefore in the present study, bile acids in bile were subjected to gas chromatography-mass spectrometry (GC-MS).

A 72 year-old female patient suffering from obstructive jaundice and treated with percutaneous transhepatic cholangiodrainage was the subject of the study. Two weeks after the external bile duct drainage, 300 mg of DHCA (1.5 ml of 20% Choleretin®, Dainippon Pharm. Co., Japan) was given intravenously. In animal experiment male Wistar strain rats, weighing about 300 g, were anesthetized with ether and the common hepatic duct was cannulated with a PE-50 tube (0.58 mm ID). The rats were placed in Bollman restraining cages while regaining consciousness and they received a continuous infusion of saline at a rate of 2.2 ml/h via the jugular vein throughout the experiment. Following 3 h of biliary drainage, DHCA-sodium

salt (Steraloids Inc., Wilton, NH) dissolved in saline (81.8 mM) was infused at 1 μmol/min/100 g of body weight. In both studies, bile was collected at regular time intervals before and after the administration of DHCA. Only trace amounts of secondary bile acids were detected, and no ketonic bile acid.

Bile was extracted with 20-fold volumes of ethanol at 78°C and an aliquot of the extract (about 20 μg as bile acids) was evaporated to dryness. Following enzymatic hydrolysis with cholylglycine hydrolase (E.C.3.5.1.24., Sigma Chemical Co., MO)[4] and extraction with ethyl acetate, bile acids were esterified with 5% HCl in ethanol. Prior to silylation, the keto groups were converted to O-methyloximes with methoxyamine HCl (5 mg) in dry pyridine (50 μl) at 100°C for 1 h.[5] Then the hydroxy groups were converted to dimethylethylsilyl (DMES) ethers and the derivatives were passed through the Sephadex LH-20 column.[6]

GC-MS was carried out using a Shimadzu AUTO GCMS 9020-DF (Kyoto, Japan), equipped with a data processing system (SCAP 1123), a Van den Berg's solventless injector and a fused silica capillary column (Hicap CBP1, Shimadzu), 15 m x 0.2 mm ID. The operating conditions were: injector temperature 310°C, column 275°C, separator 300°C, ionization source 310°C, helium carrier gas 40 ml/min, ionization

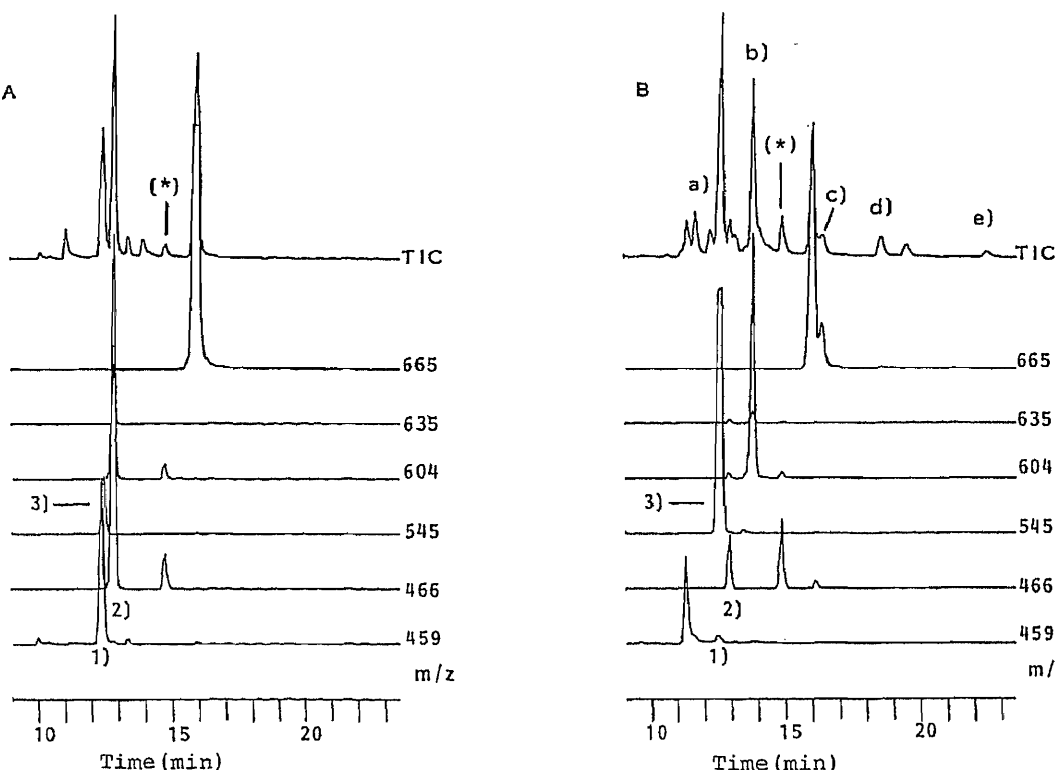

Fig.1. Selected Ion Recording of Ethyl Ester-DMES Ether-Methyloxime Derivatives of Bile Acids from Human (A) and Rat (B) Bile TIC: Total ion chromatogram. Acids: 1) chenodeoxycholic, 2) 3α,7α-dihydroxy-12-oxo-5β-cholanoic, 3) 3α-hydroxy-7,12-dioxo-5β-cholanoic, 4) cholic, a) deoxycholic, b) 3α,12α-dihydroxy-7-oxo-5β-cholanoic, c) 3α,7β,12α-trihydroxy-5β-cholanoic, d) β-muricholic, e) ω-muricholic, and (*) 3α,7β-dihydroxy-12-oxo-5β-cholanoic

energy 20 eV, trap current 60 μA. 3α,7β-Dihydroxy-12-oxo-5β-cholanoic acid and 3α, 7β,12α-trihydroxy-5β-cholanoic acid were kindly donated respectively by Dr. T. Nambara (Tohoku University, Sendai, Japan) and Dr. G.A.D. Haselwood (University of London, UK).

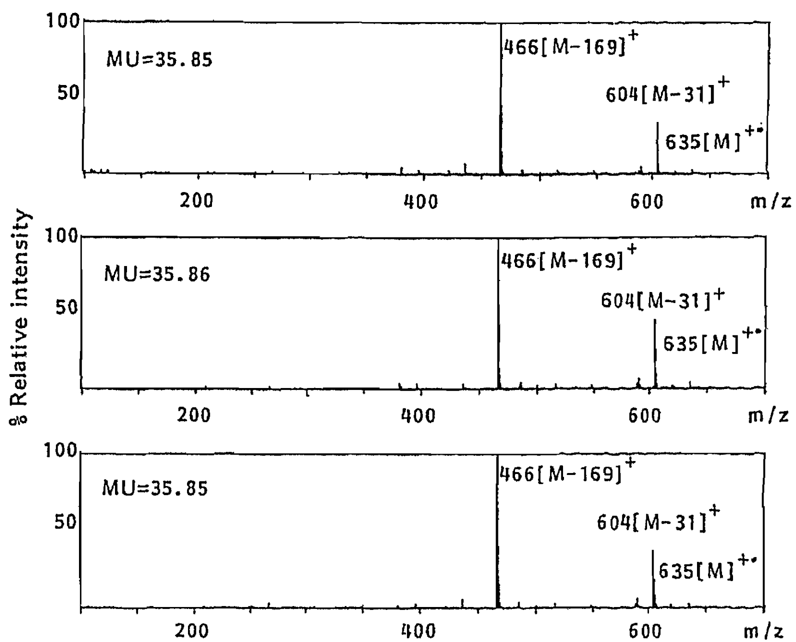

Fig.2. Mass Spectra of Bile Acids from Human (Upper) and Rat (Middle) Bile, and Authentic 3α,7β-Dihydroxy-12-oxo-5β-cholanoic Acid (Lower)
MU values are also listed

Figure 1-A shows a typical selected ion recording of the bile acid derivatives from human bile. Identification of individual bile acid derivatives was made by comparing their methylene unit (MU) values and mass spectra with those of reference compounds. The MU value and mass spectrum of peak (*) (Fig. 2 upper spectrum) were entirely identical to those of authentic 3α,7β-dihydroxy-12-oxo-5β-cholanoic acid (Fig. 2 lower spectrum). The molecular ion $[M]^{+\cdot}$ appeared at m/z 635. The fragment ion at m/z 604 $[M-31]^{+}$ resulted from a loss of $-OCH_3$, and an ion at m/z 590 $[M-45]^{+}$ from a loss of $=N-OCH_3$. A base ion at m/z 466 $[M-169]^{+}$ represents the loss of a D-ring and side chain which is typical of bile acids with oxime at C-12.[7] An identical result was observed in rats (Fig. 2 middle spectrum) and a significant amount of 3α,7β,12α-trihydroxy-5β-cholanoic acid was identified in the bile (Fig. 1-B, peak c: a base ion at m/z 665, MU=36.33).

In the present study 7β-hydroxy metabolites of DHCA, their occurrence in rats having so far been only speculated, were identified in both the human and rats, indicating that human and rat livers can reduce DHCA not only to 7α- but also to 7β-hydroxy forms. It may be that similar changes occur under various pathologic conditions.

As the configuration of the hydroxy group at C-7 strongly affects the physicochemical properties of bile acids, such as bile flow, regulation of the bile acid synthesis rate, cytotoxicity, membrane disturbance, cholesterol and phospholipids solubility and critical micellar concentration, further study is needed to clarify the physiological significance of the hepatic reduction of DHCA.

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Communications to the Editor

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DIETHYL 1-FLUORO-1-PHENYLSULFONYLMETHANEPHOSPHONATE, A VERSATILE AGENT FOR THE PREPARATION OF MONOFLUORINATED BUILDING BLOCKS

Toru Koizumi,* Toru Hagi, Yoshiharu Horie, and Yoshio Takeuchi
Faculty of Pharmaceutical Sciences, Toyama Medical & Pharmaceutical University, Sugitani 2630, Toyama 930-01, Japan

Diethyl 1-fluoro-1-phenylsulfonylmethanephosphonate was prepared and was transformed into 1-fluoroalkanephosphonate and phenyl 1-fluorovinyl sulfones, which are useful monofluorinated building blocks.

KEYWORDS—— diethyl 1-fluoro-1-phenylsulfonylmethanephosphonate; 1-fluoroalkanephosphonate; 1-fluorovinyl sulfone; monofluorinated building block; cycloaddition; Michael reaction; Emmons-Horner reaction

Monofluorinated derivatives of biologically active compounds have recently received increasing interest from the viewpoint of synthetic organic and medicinal chemistry.[1] The direct introduction of a fluorine atom into organic compounds is , however, rather difficult because the fluorination methods so far reported need drastic conditions and are not selective enough. Thus, the widely useful monofluorinated building blocks are greatly needed. For this purpose, we have noticed the 1-fluoro-1-sulfur-containing methanephosphonates as potential and promising agents because they might provide various kinds of building blocks using the characteristic chemical properties of sulfur and phosphorus atoms.
The synthetic versatility of the methanephosphonates functionalized with sulfur at the alpha position is well known.[2] However, there have been no reports on synthesizing 1-fluoro-1-sulfur-functionalized methanephosphonates.

Here we present the preparation of diethyl 1-fluoro-1-phenylsulfonylmethane-phosphonate and its conversion to 1-fluoroalkanephosphonates and phenyl 1-fluoro-vinyl sulfones, both of which are potentially useful for the preparation of various kinds of monofluorinated organic compounds.

The diethyl esters of phenylsulfonyl- (**1**), phenylsulfinyl- (**2**), and phenylsulfenylmethanephosphonates (**3**) were fluorinated using freshly prepared perchloryl fluoride ($FClO_3$)[3] in the presence of a base (KH) in THF. Sulfone 1 was effectively mono-fluorinated to afford diethyl 1-fluoro-1-phenylsulfonylmethane-phosphonate (**4**)[4] in 50-60% isolated yield, along with a small amount of the starting material. But the same fluorination reaction of 2 and 3 did not yield the expected monofluorination products in satisfactory yields (29% for 2 and 0% for 3).

$$\underset{\mathbf{1}(n=2),\mathbf{2}(n=1),\mathbf{3}(n=0)}{PhS(O)_nCH_2PO(OEt)_2} \longrightarrow \underset{\mathbf{4}(n=2)}{PhS(O)_nCHFPO(OEt)_2}$$

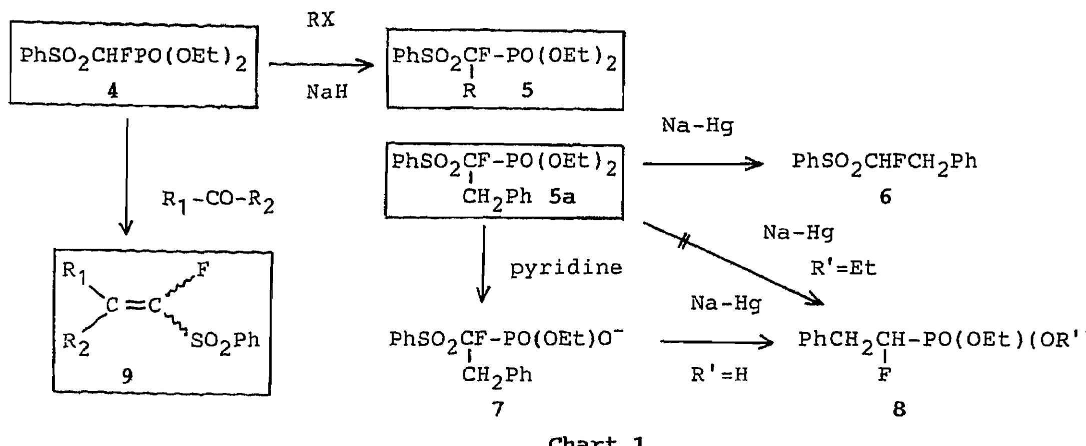

Chart 1

Compound **4** was successfully alkylated using benzyl bromide and NaH in DMF to give diethyl 1-benzyl-1-fluoro-1-phenylsulfonylmethanephosphonate (**5a**) in 99% yield. The reaction of **4** with other primary alkyl halides (RX) proceeded similarly to give the corresponding products (**5**) in fair to good yields: ethyl (RX:EtI, 69%), allyl (RX:CH_2=$CHCH_2Br$, 66%), phenethyl (RX:$PhCH_2CH_2Br$, 66%), and p-nitrobenzyl (RX:p-$NO_2C_6H_4CH_2Br$, 96%) (Chart 1).

As **4** was successfully alkylated to afford diethyl 1-alkyl-1-fluoro-1-phenylsulfonylmethanephosphonate **5** in good yields, we attempted to convert **5** to 1-fluoroalkanephosphonates, which are rather difficult to obtain by the existing fluorination methods.[5] The desulfurization of compound **5a** using Raney-Ni did not afford the expected diethyl 1-fluoro-2-phenylethanephosphonate but afforded the starting material unchanged. The sodium-amalgam treatment (Na-Hg, Na_2HPO_4, EtOH-THF)[6] of **5a** did not provide the desulfurization product but, surprisingly, afforded 1-fluoro-2-phenylethyl phenyl sulfone (**6**) in ca 30% yield. The same anomalous P-C bond cleavage under the basic conditions occurred in the reactions of **4**. The unusual instability of the P-C bond in compounds **4** and **5** to the base-catalyzed solvolytic conditions could be attributed to the high electrophilic reactivity of the phosphoryl center due to the multifunctional structure of these compounds containing a sulfonyl group and a fluorine atom.

As the diester **5a** afforded the C-P bond cleavage product by the desulfurization reaction under the basic conditions, the desulfurization reaction of ethyl monohydrogenphosphonate (**7**) was undertaken because in this compound the nucleophilic attack on phosphorus should be much more difficult. The nonsaponificative hydrolysis of compound **5a** using pyridine (heated at 80°C for 15 h) afforded N-ethylpyridinium monoethyl hydrogenphosphonate **7** quantitatively. The Na-Hg treatment (Na-Hg, Na_2HPO_4, EtOH/THF) of compound **7** gave in 72% yield the desulfurization product, 1-fluoro-2-phenylethanephosphonic monoester **8**(R'=H). The above results indicate that compound **4** is a good starting material for the preparation of various kinds of 1-fluoroalkanephosphonates.

Next we turned our attention to the use of the phosphonate **4** as an Emmons-Horner-type reagent for the preparation of 1-fluorovinyl sulfones. Phenyl vinyl sulfone is well known as useful ethylene and terminal olefin equivalents in Diels-Alder cycloadditions.[7] As an extension, Kobayashi et al.[8] recently introduced 1-phenylsulfonyl-3,3,3-trifluoropropene as a good Michael acceptor and a good dienophile. On this basis, we considered that phenyl 1-fluorovinyl sulfone derivatives could be useful as Michael acceptors and dienophiles to afford various kinds of monofluorinated organic compounds.
The Emmons-Horner reaction of **4** with several carbonyl compounds proceeded smoothly to give the fluorovinyl sulfones **9** in fair to good yields. As a typical example, the reaction with formaldehyde is described and all the results are shown in Table I. To a stirred mixture of 6.00 mmol of NaH in THF at -70 °C, a solution of phosphonate **4**(5.00 mmol in THF) was added and stirred for 2 h. To this solution was added 20 mmol of paraformaldehyde and the reaction mixture was kept at 0 °C for 1-2 h. After the usual work-up and the column chromatography, phenyl 1-fluorovinyl sulfone **9a** was obtained in 70% yield.

Table I. Emmons-Horner Reaction of **2** with Carbonyl Compounds

Carbonyl compounds	1-Fluorovinyl sulfones yield	E/Z ratio	Carbonyl compounds	1-Fluorovinyl sulfones yield	E/Z ratio[9]
HCH=O	70 %	-	$CH_3CH_2CH_2CH$=O	71 %	1
PhCH=O	85 %	E only	p-NO_2-C_6H_4CH=O	95 %	14
p-MeO-C_6H_4CH=O	81 %	49	O=CH-$COOCH_2$Ph	62 %	1.3
CH_3COCOOEt	86 %	0.7	MeCOMe	71 %	-
PhCOMe	74 %	24	Cyclohexanone	92 %	-
Cinnamaldehyde	72 %	E only	β-ionone	64 %	2.3

To elucidate the reactivity of 1-fluorovinyl sulfones in the Michael and Diels-Alder addition reactions, preliminary experiments were carried out using 1-fluorovinyl sulfone **9a** (Chart 2).
The Michael reaction of **9a** with thiophenolate and the carbanion of diethyl acetamidomalonate proceeded smoothly to afford the Michael adducts **10** and **11** in 88% and 86% yields, respectively. The adduct (**11**) was desulfurized with Na-Hg to give monofluoroethyl derivative **12** in 40% yield.
The Diels-Alder cycloaddition of **9a** with anthracene occurred in benzene solution at 150 °C for 142 h to give the cycloadduct (**13**) in 33% yield along with the recovered starting material (23%). The cycloaddition of **9a** with a large excess of cyclopentadiene at 40°C for 14 h afforded both endo and exo cycloadducts **14** in 1:1 ratio, quantitatively.[10] These results clearly indicate that 1-fluorovinyl sulfones **9**, synthetic equivalent to fluoroethenes, are reactive enough in Michael and Diels-Alder reactions to give various kinds of monofluorinated organic compounds.

The application of the monofluorinated building blocks **4**, **5**, and **9** to the preparation of biologically active substances is now in progress in this laboratory and will soon be reported elsewhere.

Chart 2

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4) Typical procedure for the monofluorination is as follows. A mixture of 1 (3.7 mmol) and KH (4.0 mmol) in dry THF (40 ml) was stirred at 0-15°C for 2 h. To the mixture, $FClO_3$ gas was introduced under nitrogen for 0.5-1 h. Ordinary workup followed by silica gel column chromatography afforded 4, mp 64 °C (hexane-Et_2O). All new compounds in this paper were fully characterized spectroscopically (IR, H-NMR, mass specta) and by combustion and/or high resolution mass spectral analysis.

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9) The E/Z ratio was determined by the 19F NMR.

10) Equal formation of the endo and exo sulfones indicates that the fluorine atom exhibits the same endo selectivity as the phenylsulfonyl group.

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Communications to the Editor

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STRUCTURE OF SCOPARIC ACID A, A NEW LABDANE-TYPE DITERPENOID FROM A PARAGUAYAN CRUDE DRUG "TYPYCHÃ KURATŨ" (*SCOPARIA DULCIS* L.)

Masaru Kawasaki,[a] Toshimitsu Hayashi,*,[a] Munehisa Arisawa,[a]
Mineo Shimizu,[a] Shunji Horie,[a] Hajime Ueno,[a] Hisashi Syogawa,[a]
Shoichi Suzuki,[a] Masao Yoshizaki,[a] Naokata Morita,[a]
Yasuhiro Tezuka,[b] Tohru Kikuchi,*,[b]
Luis H. Berganza,[c] Esteban Ferro,[c] and Isabel Basualdo[c]
Faculty of Pharmaceutical Sciences[a]
and Research Institute for Wakan-Yaku (Oriental Medicines)[b]
Toyama Medical and Pharmaceutical University,
Sugitani 2630, Toyama 930-01, Japan and
Facultad de Ciencias Qũimicas, Universidad Nacional de Asunciõn,[c]
Casilla de Correo 1055, Asunciõn, Paraguay

A new labdane-type diterpenoid, scoparic acid A, which has an inhibitory effect on β-glucuronidase, was isolated from *Scoparia dulcis* L. and its structure was elucidated by means of 2-D NMR spectroscopy including the INADEQUATE and ^{1}H- ^{13}C long-range COSY.

KEYWORDS —— scoparic acid A; diterpenoid; labdane; Typychã Kuratũ; *Scoparia dulcis* ; Scrophulariaceae; β-glucuronidase inhibitor; 2-D INADEQUATE; ^{1}H-^{13}C long-range COSY

"Typychã Kuratũ", whole plants of *Scoparia dulcis* L., (Scrophulariaceae), is one of the Paraguayan folk medicines often used for the treatment of stomach disease as well as for hepatosis. From the roots of this plant, 6-methoxybenzoxazolinone,[1)] having a hypertensive activity, has been isolated by Taiwan workers. In the course of our search for biologically active substances from Paraguayan medicinal plants, we found that the extract of "Typychã Kuratũ" has a great inhibitory effect on β-glucuronidase. By bioassay-guided separation, we isolated a labdane-type diterpenoid, named scoparic acid A (1), which inhibited the activity of β-glucuronidase from bovine liver (IC_{50},

1 R^1=R^2=H ,R^3=COC_6H_5
2 R^1=CH_3, R^2=H, R^3=COC_6H_5
3 R^1=H, R^2=$COCH_3$, R^3=COC_6H_5
4 R^1=R^2=R^3=H

$6.8X10^{-6}M$). It also mildly inhibited histamine-induced contraction of the ileum isolated from guinea pigs (IC_{50}, $3.2X10^{-5}M$). In this communication, we report the structure of 1.

The aqueous ethanol (70%) extract from dried "Typychã kuratũ" was partitioned between n-hexane and water. The precipitate obtained from the aqueous layer was chromatographed on a silica gel column using a MeOH-$CHCl_3$ stepwise gradient and a fraction eluted with MeOH-$CHCl_3$(1:9) was further purified by HPLC on a TSK-GEL Silica 60 column with the MeOH-$CHCl_3$ stepwise gradient (1:99 to 10:90) to give scoparic acid A (1) as a colorless amorphous powder.

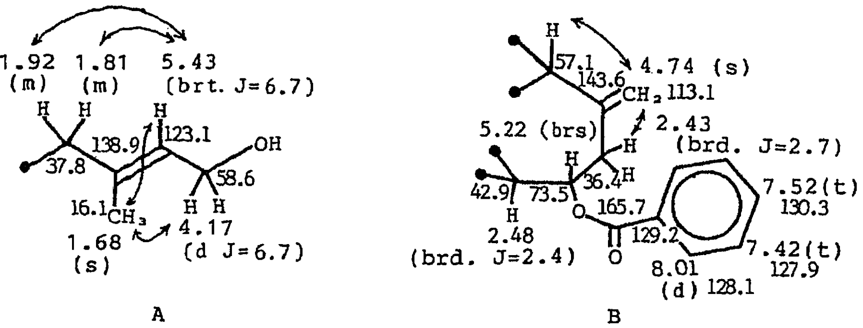


Fig.1. Partial Structures of Scoparic Acid A (δ-Values in $CDCl_3$)
(↗ long-range coupling in $^1H-^1H$ COSY)

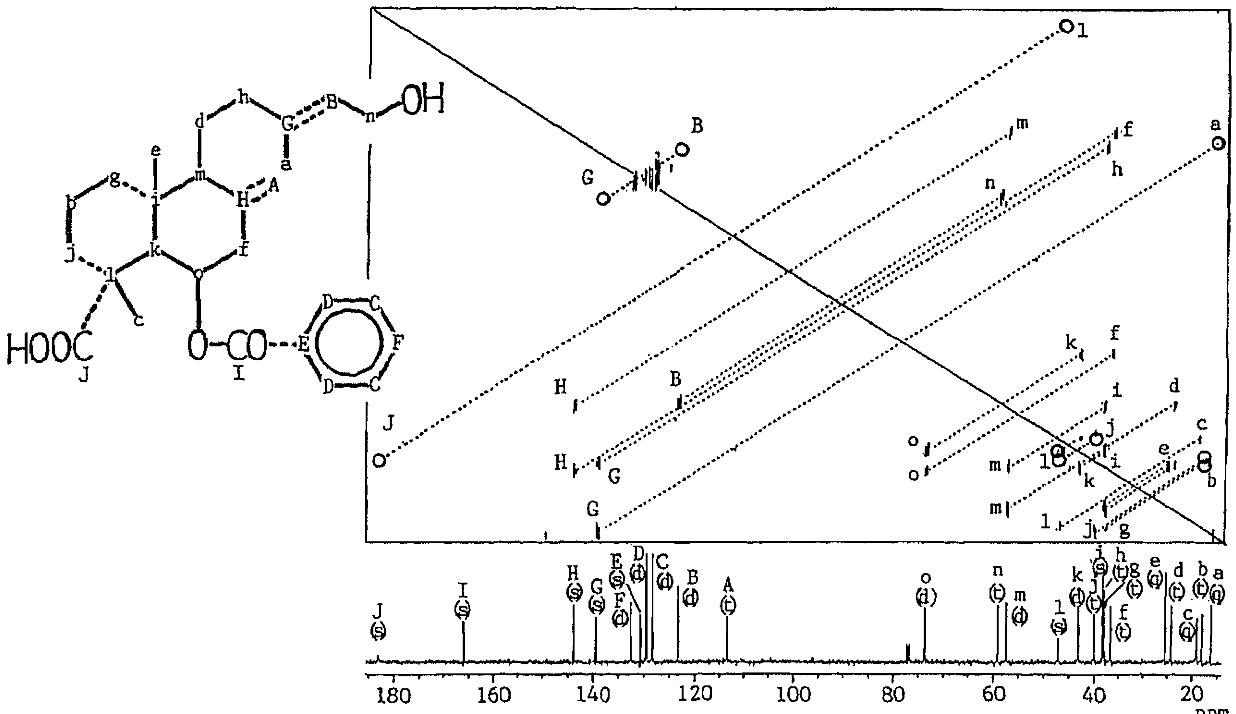
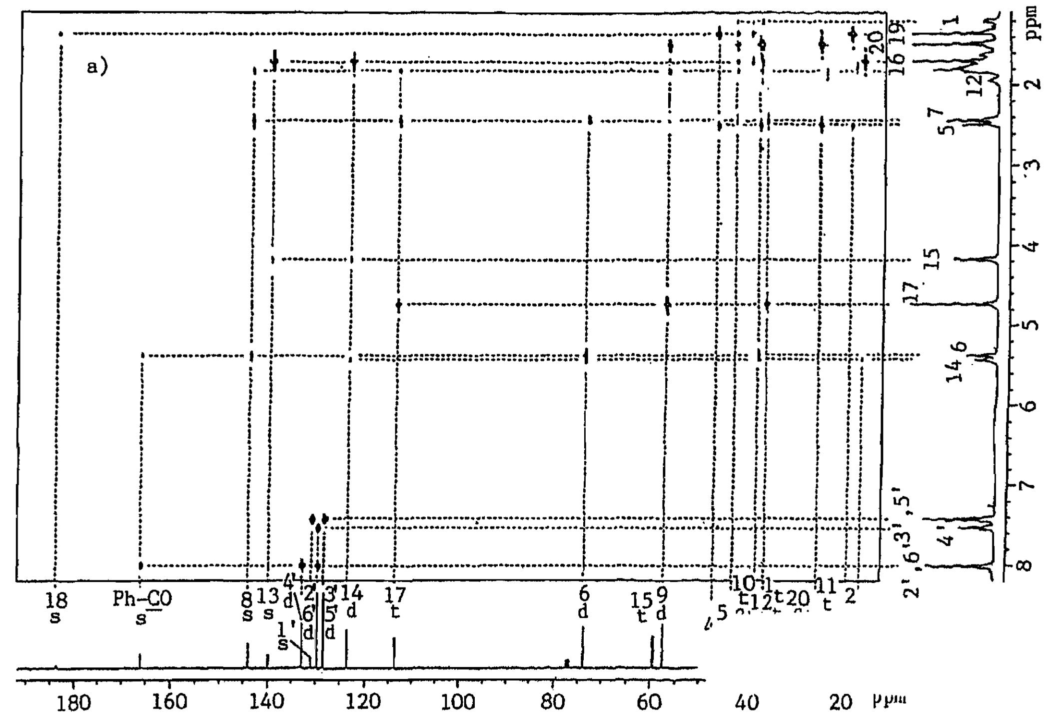


Fig.2. 2-D INADEQUATE Spectrum (Contour Map) of Scoparic Acid A (1) in $CDCl_3$

The spectrum was measured on a JEOL GX-400 spectrometer, using 170 mg of the sample (40°C, 60h run, J_{CC}=45Hz). The multiplicities of carbon signals were determined by the off-resonance and DEPT methods and indicated as s, d, t and q; sp^2 carbons are marked with A-J and sp^3 carbons with a-o in the order of increasing δ values. Open circles indicate the expected carbon signals, which were not observed in this measurement, but were observed in other experiments.

Scoparic acid A (1), $C_{27}H_{36}O_5$, $[\alpha]_D$ -38.3°($CHCl_3$), showed IR absorptions at 3400(OH), 1700(CO), 1600 and 1580 cm^{-1} (phenyl) and UV absorptions at 227, 265(sh), 270 and 277 nm. The EI-MS of 1 showed the molecular ion peak at m/z 440 and fragment ion peaks at m/z 422 (M^+-H_2O), 394 (M^+-HCOOH), 377 (M^+-H_2O-COOH), 335 (M^+-C_6H_5CO), 318 (M^+-C_6H_5COOH), 300, 173 ($C_{13}H_{17}^+$), 159 ($C_{12}H_{15}^+$), 105 ($C_6H_5CO^+$) and 77 ($C_6H_5^+$). The ^1H-NMR spectrum showed signals due to one vinyl methyl (δ 1.68), two tertiary-methyls (δ



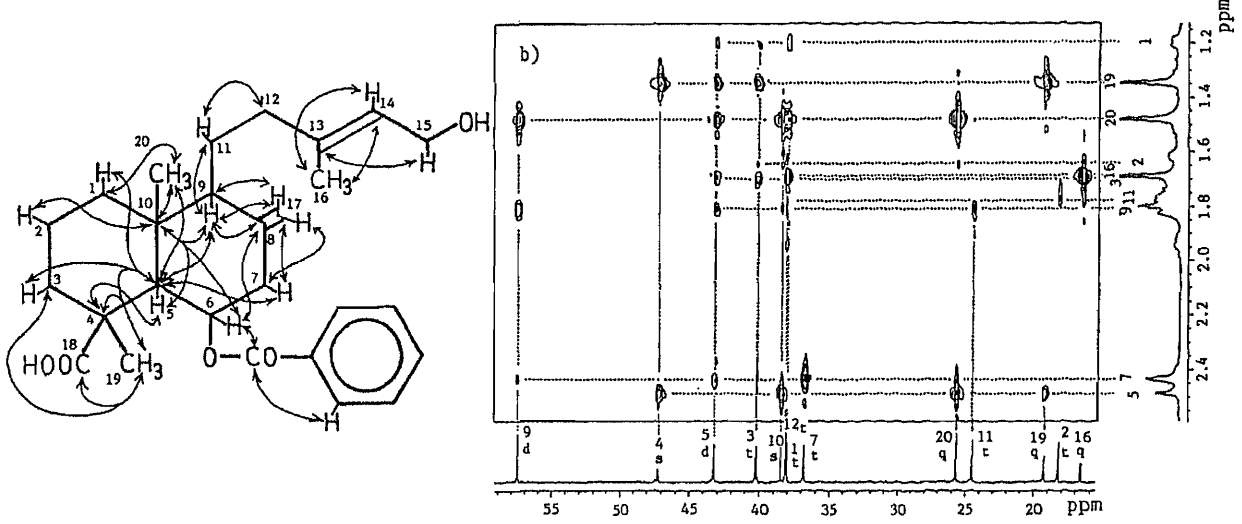


Fig.3. ^1H-^{13}C Long-Range Shift Correlation Spectra of Scoparic Acid A in $CDCl_3$:
a) Whole region. b) High field region

The spectra were measured using 90mg of the sample (35°C,12 h run, J_{CH}=10Hz). The ^1H-signals were assigned using ^1H-^1H and ^1H-^{13}C shift correlation spectra.

1.34 and 1.47), one carbinyl methylene (δ 4.17) and one phenyl group (δ 7.42, 2H, 7.52, 1H and 8.01, 2H), along with three olefinic protons (δ 4.74, 2H and 5.43, 1H). On treatment with diazomethane and with acetic anhydride-pyridine, **1** gave a methyl ester (2), $C_{28}H_{38}O_5$, $[\alpha]_D$ -50.2°($CHCl_3$), δ 3.69 ($COOCH_3$), and an acetate (3), $C_{29}H_{38}O_6$, δ 4.62 (d, J=7.1, $C\underline{H}_2OAc$), 2.07 ($OCOC\underline{H}_3$), respectively.

Scoparic acid A resisted hydrolysis by the usual method but was hydrolyzed by heating with 4% KOH in MeOH-DMSO in a sealed tube yielding a debenzoylated compound (4), $C_{20}H_{32}O_4$, δ 4.00 (H-6).

These data and detailed ^1H- and ^{13}C-NMR studies of 1 with the aid of ^1H-^1H and ^1H-^{13}C shift correlation spectroscopy led us to conclude that 1 may be a diterpene acid having the partial strucure A and B (Fig. 1).

At this stage, the 2-D INADEQUATE spectra[2] of **1** were measured under various conditions to determine the carbon-carbon connectivities in the molecule. As illustrated in Fig. 2, there were correlation peaks of all the coupled ^{13}C-^{13}C pairs except those between the carbons g and i, A and H, and E and I. Therefore a labdane-type bicyclic structure was deduced for **1**.

Next, we measured the ^1H-^{13}C long-range shift correlation spectrum of **1** in order to confirm the sequence of carbon atoms. As shown in Fig. 3, the methylene carbon at δ 38.0(C-1) is correlated with the methyl protons at δ 1.48 (20-H_3). In turn, the carbon atoms corresponding to the signals at δ 38.0 (C-10) and at δ 42.9 (C-5) are correlated with the protons corresponding to the signals at δ 1.48 (20-H_3), 1.61 (2-H), 1.81 (9-H), 2.50 (5-H) and 5.38 (6-H) and at δ 1.19 (1-H), 1.35 (19-H_3), 1.48 (20-H_3), 1.70 (3-H), 1.81 (9-H) and 2.44 (7-H_2), respectively. Also, some of significant ^1H-^{13}C long-range correlations are indicated by arrows in the formula in Fig. 3. Thus, the planar structure of this compound was shown to be **1**.

The relative stereochemistry of scoparic acid A (**1**) was determined by nuclear Overhauser effect (NOE) difference spectroscopy. Irradiation of the 19- and 20-methyls enhanced the signal intensity of the 20- and 2',6'-protons and the 19-and 2',6'-protons respectively. Similarly, irradiation of the 16-methyl increased the signal intensity of the 15-metylene protons. Also, NOE's were observed between 5- and 6-protons and between the 6- and 7-protons. Thus, the relative configuration of scoparic acid A was determined as represented by the formula **1**.[3]

Details of the biological activities of scoparic acid A will be reported elsewhere.

AKNOWLEDGMENT This work was supported in part by a grant from Japan International Cooperation Agency and a Grant-in-Aid for Scientific Reserch to T.K. (No.61470147) from the Ministry of Education, Science and Culture, Japan.

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Communications to the Editor

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RADICAL DEOXYGENATION OF TERT-ALCOHOLS IN 2'-BRANCHED-CHAIN SUGAR PYRIMIDINE NUCLEOSIDES: SYNTHESIS AND ANTILEUKEMIC ACTIVITY OF 2'-DEOXY-2'(S)-METHYLCYTIDINE[1)]

Akira Matsuda,[*, a] Kenji Takenuki,[a] Hiroko Itoh,[a] Takuma Sasaki,[b] and Tohru Ueda[a]

Faculty of Pharmaceutical Sciences, Hokkaido University,[a] Kita-12, Nishi-6, Kita-ku, Sapporo 060 and Cancer Research Institute, Kanazawa University,[b] Takara-machi, Kanazawa 920, Japan

We have synthesized 2'-deoxy-2'(S)-methylcytidine (7), a new antileukemic nucleoside. The carbonyl methylation of 2'-ketonucleoside (1) with MeLi, Me_3Al and MeMgX was examined. Only in the reaction with MeMgX, did the more hindered β-attack afford the 2'-methyl-t-alcohol (2b). Compound 2b was converted into the methyl oxalate (4), which was subjected to radical deoxygenation to give the 2'-deoxy-2'(S)-methyl derivative (5). The deprotection of 5 followed by substitution with NH_3 furnished 7. The structure-activity relationships of 7 and some other 2'-branched-chain sugar cytidines against L1210 cells are also described.

KEYWORDS——carbonyl methylation; radical deoxygenation; nucleoside; branched-chain nucleoside; 2'-deoxy-2'(S)-methylcytidine; antileukemic activity

Introducing of a substituent into the C-2' position with the arabino configuration from a naturally-occurring pyrimidine ribonucleoside is extremely difficult, because the C-2 carbonyl group of the pyrimidine base is so close to the C-2' leaving group in the ribo configuration of the sugar residue that O^2,2'-cyclonucleosides is formed instead.[2)] Although several 2'-substituted-arabinofuranosyl pyrimidine nucleosides have been found to be potent antimetabolites,[3)] all of these nucleosides have been synthesized by the condensation of the nucleobases with appropriately substituted sugar previously obtained by multi-step manipulations. This method usually gives an anomeric mixture and requires tedious separation processes. Although certain branched-chain sugar nucleosides[4)] have shown interesting biological activities, they have also been synthesized by the classical condensation methods. Here, we describe a new method for the synthesis of 2'-deoxy-2'-branched-chain sugar pyrimidine nucleosides with the carbon chain of the sugar branches in the arabino configuration, from the naturally occurring pyrimidine ribonucleoside uridine. This method consists of the carbonyl alkylation of the 2'-ketonucleosides and subsequent radical deoxygenation of the methyl oxalyl ester of the tert-alcohols.

OEt N O N O TIPDS O O O

1

2a

2b

3a

3b

B=4-ethoxy-2(1H)-pyrimidinon-1-yl

4

5

6

7

8: X=H

9: X=$COCO_2Me$

10: R=Ac

11: R=H

12

13

14

15

The protected 2'-ketonucleoside (1), as a key substrate, was prepared in five steps from uridine. When 1 was treated with MeLi in THF at -78°C or with Me_3Al in $CHCl_3$ at -50°C, the sole product obtained in 88% or 82% yield, respectively, was the undesired 2'(S)-methyl derivative (2a). From these results and previous observations,[5] it appears that the α-face of the sugar in the 2'-ketonucleoside is less hindered. While investigating the introduction of a methyl substituent to the more hindered β-position, we found that the treatment of compound 1 with MeMgBr (or MeMgI, 5 eq.) in Et_2O at -50°C, gave the desired 2'(R)-methyl-*tert*-alcohol (2b) in 43% yield along with 2a in 50% yield. To improve the β-stereoselectivity, methylaluminum bis(2,4,6-*tert*-butylphenoxide),[6] which might form a stable complex with the carbonyl oxygen at the 2'-position, was employed with MeMgBr. However, inverse selectivity afforded the undesired nucleoside (2a) instead. Also, the use of $BF_3 \cdot OEt_2$ did not improve the stereoselectivity.

The C-2' configuration of these nucleosides (2a,b) was determined as follows. Removal of the silyl protecting group by fluoride ions in THF gave the free nucleosides (3a,b),[7] respectively. The free nucleoside (3b) bearing the *ribo* configuration showed a positive periodate-benzidine spray test. Compound 3b formed a 2',3'-cyclic borate complex and migrated on paper electrophoresis, but 3a did not.

The *tert*-alcohol in 2b was deoxygenated by the method developed by Dolan and MacMillan.[8] The 2'(R)-methyl-*tert*-alcohol (2b) was treated with methyl oxalyl chloride in the presence of dimethylaminopyridine (DMAP) to afford the ester (4), which was then subjected to radical deoxygenation with Bu_3SnH and AIBN in hot toluene. The reaction proceeded smoothly to give exclusively the desired 2'-deoxy-2'(S)-methylnucleoside (5). However, 2'-(S)-*tert*-alcohol (2a) resisted reacting with methyl oxalyl chloride even under vigorous conditions, probably due to the steric hindrance of the 3',5'-protecting group. Therefore, compound 3a was converted to its di-O-acetate (8) by treatment with Ac_2O and Et_3N in the presence of DMAP in acetonitrile.[9] The methyl oxalate (9) was easily obtained from 8. The ester 9 was deoxygenated under similar conditions giving a product which showed a single spot on thin layer chromatography. However, the ^1H-NMR spectrum of the product indicated that this was a mixture of 2'-deoxy-2'(R)- and (S)-isomers (10,12) in a ratio of 1:3. It is obvious that Bu_3SnH as a reductant reacts preferably from the less hindered α-face of the *tert*-alkyl radical intermediate. This epimeric mixture was separated, after deprotection by treatment with NH_3/MeOH at room temperature, by semi-preparative reverse phase high pressure liquid chromatography to give 6 and 11, respectively. The stereochemistry at the 2'-position was determined by NOE experiments in which the NOE (5.4%) are observed between the H-6 and 2'(S)-methyl protons in 6.

The title compound 2'-deoxy-2'(S)-methylcytidine (7)[10] was obtained by treatment of 6 with NH_3/MeOH in a sealed tube at 100°C. Other 2'-branched-chain sugar nucleosides were also converted to the corresponding cytosine derivatives (13-15)[11] in a similar manner.

Among these 2'-branched-chain sugar cytosine nucleosides, compound 7 was the most active against mouse leukemic cell line L1210 cells (IC_{50} = 0.26 μg/ml). The order of the inhibitory activity against the cells *in vitro* was 7,

15, 13 and 14. It is of interest to note that although compound 14 is an analog of 1-β-D-arabinofuranosylcytosine, a prominent antileukemic drug in clinical use, it showed only 14% inhibitory activity even at 400 μg/ml, while cytidine analog 15 had an IC_{50} value of 15 μg/ml.

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10) 7 was isolated as HCl salt, mp 167-169°C (EtOH). ^{1}H-NMR (D_2O) δ: 0.95 (3H,d,2'-Me), 2.74 (1H,m,2'-H), 3.89-4.06 (4H,m,3',4',5'-H), 6.24 (1H,d,5-H), 6.29 (1H,d,1'-H,J=7.6Hz), 8.13 (1H,d,6-H).
11) 13 was isolated as HCl salt, mp 183-186°C (dec. MeOH-acetone). ^{1}H-NMR (D_2O) δ: 1.08 (3H,d,2'-Me,J=7.1 Hz), 2.45 (1H,m,2'-H), 3.80 (2H,m,5'-H), 4.13 (1H,m,4'-H), 4.29 (1H,dd,3'-H), 5.94 (1H,d,1'-H,J=8.1 Hz), 6.26 (1H,d,5-H), 8.08 (1H,d,6-H). 14 was isolated as HCl salt, mp 195-197°C (MeOH). ^{1}H-NMR (D_2O) δ: 1.38 (3H,s,2'-Me), 3.90-4.04 (4H,m,3',4',5'-H), 6.01 (1H,s,1'-H), 6.23 (1H,d,5-H), 8.09 (1H,d,6-H). 15: mp 239.5-242°C (MeOH, lit[12] 243-244°C). ^{1}H-NMR (D_2O) δ: 1.12 (3H,s,2'-Me), 3.73-4.10 (4H,m,3',4',5'-H), 6.04 (1H,d,5-H), 6.06 (1H,s,1'-H), 7.85 (1H,d,6-H).
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Communications to the Editor

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PHOTOCYCLOADDITION, METHYLATION, AND CATALYTIC REDUCTION OF CHIRAL SPIROCYCLIC 1,3-DIOXIN-4-ONES: DIFFERENT STEREOFACIAL SELECTIVITY AND ITS EXPLANATION[1)]

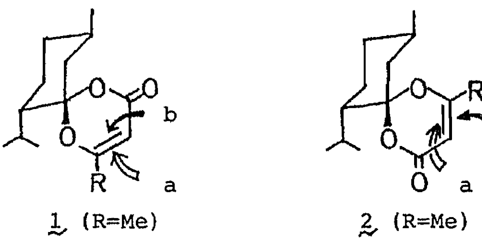
Masayuki Sato,[*,a] Kazuhisa Takayama,[a] Toshio Furuya,[b] Noriyoshi Inukai,[b] and Chikara Kaneko[*,a]

Pharmaceutical Institute, Tohoku University,[a] Aobayama, Sendai 980, Japan and Central Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd.,[b] 1-8, Azusawa 1-chome, Itabashi-ku, Tokyo 174, Japan

We describe several reactions of chiral spirocyclic 1,3-dioxinones, all of which proceed in high stereofacial selectivity. A possible reason for the different selectivities in these reactions is proposed.

KEYWORDS——asymmetric photocycloaddition; spirocycic 1,3-dioxin-4-one; stereofacial differentiation; crystal structure; (R)-3-hydroxybutyric acid; l-menthone; chiral auxiliary group

Remarkable stereofacial differentiation (e.g. a 9-fold preference for side-a to side-b in the reaction with cyclopentene at 0°C) in the photoaddition of dioxacyclohexenones (1 and 2) reported recently by Demuth et al.[2)] has prompted us to report our own observations concerning to the same photoaddition reaction of their des-methyl derivatives (3 and 4). Though our result was the same as Demuth's, methylation and catalytic hydrogenation of these dioxinones proceeded again in high stereoselectivity, but in completely reverse stereofacial selectivity (a high preference for side-b to side-a). The experimental results as well as their possible explanation are the subjects of the present paper.



a: diastereotopic face cis to the isopropyl group

b: diastereotopic face opposite to the isopropyl group

Synthesis and Structure Determination of New Spirocyclic Dioxinones (3 and 4)

Though optically pure dioxinones (3 and 4) can be synthesized from l-menthone and formyl Meldrum's acid (6) according to our general procedure,[3)] it was found that use of 2,2-dimethyl-1,3-dioxin-4-one[3)] (5) instead of 6 gave a more satisfactory result.[4)] Thus, refluxing of 5 and a 5 molar excess of l-menthone in benzene resulted in the formation of the spirodioxinone in 59% yield as a mixture

of diastereomers (3 and 4) in ca. 1:1 ratio. Separation of these by medium-pressure column chromatography [Kieselgel 60H, hexane/ether (20/1)] afforded less polar (3: mp 115-116 °C)[5,6] and more polar dioxinones (4: mp 100-101 °C).[7] The X-ray crystallography of 3 shown in Fig. 1 unequivocally determined its structure and hence that of 4.

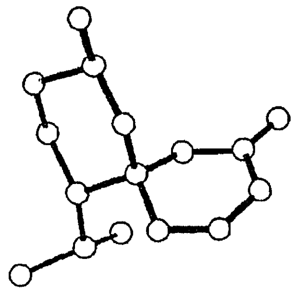


Fig. 1. Crystal Structure of (-)-3

Photoaddition of 3 and 4 to Cyclopentene

When the more polar dioxinone (4) was irradiated at 300 nm (Rayonet Photochemical Reactor RPR-3000Å, quartz vessel, room temperature) in hexane in the presence of an excess (10-20 fold) of cyclopentene, two adducts (9 and 10) were obtained in ca. 65% yield. The adducts could be separated easily by silica gel column chromatography to give the major (9: mp 107-107.5 °C) and minor adducts (10: mp 146.5-148 °C) in ca. 6:1 ratio. In the same manner, two adducts (7: mp 60-68 °C and 8: mp 84-86 °C) were also obtained from the less polar dioxinone (3) again in ca. 6:1 ratio. Since we have already demonstrated that 2,2-dimethyl-1,3-dioxin-4-one (5) and its analogues when subjected

Fig. 2. Crystal Structure of the Major Adduct (9) Obtained from 4

to the photoaddition to cyclopentene gave solely cis-anti-cis adducts,[8] these adducts (7-10) should also possess cis-anti-cis configuration at the new coupling sites. The X-ray crystallographic analysis (Fig. 2) of the major adduct (9) obtained from 4 not only realized this expectation, but also confirmed its stereostructure.

The preferential formation of 7 (or 9) is consistent with the preferred a-side addition of the dioxinones (1 and 2) reported by Demuth et al.[2] They thought that the dioxinone ring could adopt a sofa-conformation in solution just as in crystallines (c.f. Fig. 1), an arrangement that exposes the a-side to the alkene.

Methylation and Catalytic Hydrogenation of the Spirodioxinones (3 and 1)

To examine such stereofacial selectivity in other reactions, 3 was methylated by lithium dimethylcuprate in ether at -78 °C. The crude methylated product (11)[9] obtained by the usual work-up was directly subjected to mild hydrolysis (0.1N HCl-ether, room temperature) to give (R)-(-)-3-hydroxybutyric acid [(-)-12:$[\alpha]_D^{28}$ -20.1° (c=3.8, H_2O)]. Comparison of the specific rotation value with that ($[\alpha]_D^{25}$ -24.5° in H_2O)[10] of the authentic sample shows clearly that methylation occurred not from the side of isopropyl group (a-side) as in the above photoaddition reaction, but from its reverse side (b-side), with high stereofacial selectivity (d.e. 82%).

3 → 11 → (R)-(-)-12

1 → 13 → (S)-(+)-12

The preferential attack from the b-side also occurred when 1[2,11] was hydrogenated catalytically (PtO_2, MeOH, 1 atm, room temperature). The usual work-up after the hydrogenation reaction afforded directly (+)-3-hydroxybutyric acid [(+)-12: $[\alpha]_D^{28}$ +21.5° (H_2O)]. The high enantioselectivity shows again that reduction has occurred preferentially from the b-side (d.e. 88%).

Explanation of Different Stereofacial Selectivities

One reasonable explanation of the above results is to assume that those dioxinones (for examples, 1 and 3) exist in an equilibrium between two conformers (A and B), though B is surely a minor conformer. In photoaddition to cyclopentene, the major adduct (e.g. 7) should be formed via major conformer (A) by an a-side attack, as proposed by Demuth.[2] Though the b-side attack of the alkene on A is not completely denied, it is more reasonble to assume that the minor adduct (8) may be formed via the less stable conformer (B) by the attack of the alkene from the b'-side, which is far more exposed than the reverse a'-side. On the contrary, if the attac-

A ⇌ B   A' (bulky reagents)

king reagent becomes more bulky as in the case of the methylation or catalytic hydrogenation, an approach of the reagent (Me_2CuLi or H_2-PtO_2) from side-a of A should cause appreciable steric hindrance between the isopropyl group and these reagents (e.g. A') and hence, the less favorable conformer (B) accepts these reagents from the least-hindered b'-side at a much higher rate than in the a-side attack via A.

While the above explanation is still tentative, the experimental facts obtained in the present study clearly show that, though these optically active spirodioxinones (1-4) are surely useful building blocks for a variety of chiral compounds, their stereofacial selectivities depend on the reagents employed.[12]

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5) All new compounds were identified by elemental analysis and the structures were supported by acceptable spectral data.
6) 3. IR($CHCl_3$): 1730, 1620 cm^{-1}; ^{1}H-NMR($CDCl_3$)δ: 5.27 and 7.08 (each 1H, d, J=6 Hz, dioxinone ring protons; $[\alpha]_D^{24}$ -51.6° (c=1.1, $CHCl_3$).
7) 4. IR($CHCl_3$): 1730, 1620 cm^{-1}; ^{1}H-NMR ($CDCl_3$)δ:5.32 and 7.08 (each 1H, d, J=6 Hz, dioxinone ring protons; $[\alpha]_D^{25}$ -17.7° (c=1.3, $CHCl_3$).
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STEREOSELECTIVE SYNTHESIS OF 9-HYDROXYELLIPTICINE GLYCOSIDES, NOVEL AND HIGHLY ACTIVE ANTITUMOR AGENTS

Tadashi Honda,* Minako Inoue, Mika Kato,
Keiyuu Shima, and Tetsuo Shimamoto
Suntory Institute for Biomedical Research,
Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

A series of novel and highly active antitumor agents, 9-hydroxyellipticine glycosides were stereoselectively synthesized in good yields by an improved condensation reaction between 9-acetoxyellipticine and peracylated glycosyl halides using cadmium carbonate, followed by the removal of acyl groups with methanolic ammonia.

KEYWORDS——9-hydroxyellipticine glycoside; antitumor activity; stereoselective synthesis; condensation reaction; N-glycosidation; cadmium carbonate

Ellipticine (1) and 9-methoxyellipticine (2) are alkaloids originally isolated from _Ochrosia elliptica_[1] with antitumor activity against several animal and human tumors.[2] However, their poor solubility in water has led to considerable difficulty in developing formulations for clinical use. Various efforts have been made toward development of active and hydrophilic derivatives, but none were significantly better than their parent compounds.[3] Recently, 9-hydroxy-2-methylellipticinium acetate (3), a water-soluble derivative, was prepared and was found to be effective in the treatment of human breast cancer.[4]

1 R=H
2 R=OMe
9 R=OAc

3

We intended to synthesize water-soluble ellipticine derivatives with a more selective toxicity towards tumor cells by N-glycosidation of the 2-position. Consequently, 9-hydroxyellipticine glycosides (III) were found to be water-soluble with much higher activity than the parent compounds in various murine tumor systems. Herein, an efficient and stereoselective synthesis of 9-hydroxyellipticine glycosides (III) is described.

9-Acetoxyellipticine glycosides (II) can be synthesized according to the method used for the condensation of peracylated glycosyl halides (I) with nicotinamide.[5] However, this method is not satisfactory, due to low yield

and poor stereoselectivity. As a model system for improved synthesis, the condensation reaction of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (**4**) and nicotinamide was investigated.

When the mixture of nicotinamide (1 mmol) and glucosyl bromide (**4**) (1 mmol) were heated under reflux in dry nitromethane (10 ml) for 10 min, nicotinamide glucoside (**5**, α-isomer:β-isomer=12:88) was obtained in low yield (35%). The low yield of the desired glucoside (**5**) seemed to be due to the side reactions which formed products such as nicotinamide hydrobromide (**6**) (acid scavenging reaction), 2,3,4,6-tetra-O-acetyl-α-D-glucose (**7**) (hydrolysis),[6] and 2,3,4,6-tetra-O-acetyl-2-hydroxy-D-glucal (**8**) (elimination) (Chart 1).

Chart 1

We therefore postulated that the use of an acid scavenger with excess (1.5-2.0 eq) glucosyl bromide (**4**) should increase the yield of glucoside (**5**). Screening of various acid scavengers revealed that the use of $CdCO_3$ (1.5 eq) provide predominantly the desired β-glucoside (α-isomer:β-isomer=3:97) in excellent yield (99%).

Stereoselective condensation of 9-acetoxyellipticine (**9**)[3a] (1 mmol) with 2,3,4-tri-O-acetyl-β-L-arabinopyranosyl bromide (**13**)[7] (2 mmol) using $CdCO_3$ (1.5 mmol) in refluxing (10 min) dry nitromethane (30 ml) afforded 9-acetoxy-2-(2,3,4-tri-O-acetyl-L-arabinopyranosyl)ellipticinium bromide (**19**, 1',2'-trans-isomer:1',2'-cis-isomer=97:3) in 97% yield. Other peracylated glycosyl halides also gave good results. Deprotection was achieved with methanolic ammonia to give the desired 9-hydroxyellipticine glycosides (III)[8] (Chart 2 and Table).

Most of the glycosides (III) showed remarkably higher antitumor activity in

Chart 2

R=Ac, Bz; X=Cl, Br

Table. Synthesis and Antitumor Activity of 9-Hydroxyellipticine Glycosides

I[a]	II	Yield[b,c] (%)	III	Yield[b] (%)	1',2'-*trans* : 1',2'-*cis*[d]	Effect on L1210[e] Optimal dose(mg/kg)	80 days' survival
10	**16**	96	**23**	77	95 : 5	30	1 / 6[f]
11	**17**	77	**24**	80	100 : 0	10	0 / 6
12	**18**	74	**25**	94	100 : 0	30	4 / 6
13	**19**	97	**26**	87	97 : 3	30	5 / 6
14	**20**	95	**27**	84	97 : 3	30	3 / 6
4	**21**	58	**28**	75	96 : 4	10	0 / 6
15	**22**	91	**29**	79	98 : 2	20	4 / 6

a) For preparation, see footnote 7.
b) Isolated yield.
c) Isomer ratios of II were almost equal to those of III.
d) Determined by HPLC analysis.
e) Antitumor activity was assayed according to the established protocols of the National Cancer Institute.
f) Survival mice/test mice.
Cf. Activity of 3: 80 days' survival mice were not observed (0/6) at optimal dose (5 mg/kg).

various murine tumor systems (L1210, P388, B16, Colon 38 etc.). Their effects on L1210 are summarized in the Table. L-Arabinopyranoside (26) is the most promising of these antitumor agents.[9]

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6) Due to the trace amount of H_2O (0.01-0.02% by Karl Fischer's titration) in nitromethane.
7) Glycosyl halides (I) can be prepared from the corresponding parent sugars by acylation, followed by treatment with HBr or HCl, e.g. 15: R. G. Hansen, W. J. Rutter, and P. Krichevsky, Biochem. Prepar., 4, 1 (1955).
8) All the glycosides were well characterized by their physicochemical data: e.g. 2-α-L-arabinopyranosyl-9-hydroxy-5,11-dimethyl-6H-pyrido[4,3-b]carbazolium bromide (1',2'-trans-isomer of 26): mp 240-245°C (dec.); $[\alpha]_D^{18}$ -213° (c 0.15, H_2O); IR (KBr) 3250, 1640, 1600, 1480, 1420, 1220, 1200, 1150, 1090, and 1060 cm^{-1}; UV (EtOH) 227 nm (ε 14000), 268 nm (ε 24000), 281 nm (ε 21000), and 325 nm (ε 47000); MS (SIMS) m/z 395 $[C_{22}H_{23}N_2O_5]^+$; ^{1}H-NMR (360 MHz, DMSO-d_6) δ: 2.85 (3H, s, 5-CH_3), 5.74 (1H, d, J=8.5 Hz, 1'-H), 7.18 (1H, dd, J=2 Hz and 9 Hz, 8-H), 7.55 (1H, d, J=9 Hz, 7-H), 7.86 (1H, d, J=2 Hz, 10-H), 8.51 (2H, brs, 3-H and 4-H), 9.45 (1H, brs, 9-OH), 10.03 (1H, s, 10-H), and 12.08 (1H, brs, 6-H); Anal. Calcd for $C_{22}H_{23}N_2O_5Br\cdot 2H_2O$: C, 51.67; H, 5.32; N, 5.48. Found: C, 51.71; H, 5.06; N, 5.37.
9) Details of antitumor activity will be reported elsewhere.

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